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Optimization of Biofertilizers Enriched in N by Diazotrophic Bacteria

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Abstract
Free-living diazotrophic bacteria can enrich organic biofertilizer in nutrients to application in sustainable agriculture. The objective of the study was to select diazotrophic bacteria isolated from different soils, evaluated by analyses of nitrogenase activity. The diazotrophic were submitted to gene rRNA 16S sequenced and the best isolates compared with strains used to produce the biofertilizer. The biofertilizers used earthworm compost and sugarcane mud cake mixed with PK rock biofertilizer in different proportions (3:2:1 and 2:3:1). The experiment conducted in a factorial 6x2x2, used six diazotrophic bacteria applied in two concentrations (100 and 200 mL) and organic matter in the selected proportions, with four replicates. Samples were collected in times 0; 10; 20; 30 and 40 days and analyzed: pH (H_2O), total C, total N, available P and K. The rRNA 16S gene sequence identified the selected diazotrophic as *Bacillus, Mesorhizobium, Paenibacillus* and *Beijerinckia*. The strains increased nutrients in the biofertilizers, especially the isolate NFB 4 and strain NFB 1003, in incubation time of 25 and 30 days. The more effective diazotrophic increased nutrients, especially N in biofertilizer produced in proportion 3:2, and promoted significant interaction for all analyzed parameters, except to total C. Available P and K increased when applied in both proportions. The selected diazotrophic bacteria produce biofertilizers that may be used as alternative substrate to replacement of soluble fertilizer.

Keywords: Biofertilizers; free-living diazotrophic; nutrient enrichment; organic matters; rock biofertilizers; sugarcane mud cake

1. Introduction

The crescent growth of the world population and the demands for fertilizers have led to sensible changes in agricultural systems and intensify the use of new techniques to obtain the maximum yields of economical plants (Goy et al. 2009). The advances in sustainable agriculture introduce new technologies into the systems with the aim to increase available nutrients availability (Stamford et al., 2017).

Fertilization is one of the most important factors that affect yield and quality of plant products and furthermore, the increase of available nutrients in soil is necessary for sustainability and to the maximization of the agricultural crop system, especially in tropical soils (Stamford et al. 2017, 2014, 2009, 2008, 2007, 2006).

Several studies, carried out with Brazilian soils from different regions showed that the application of the phosphate rock biofertilizer, in comparison with the conventional fertilizers, promotes plant growth with higher shoot dry matter and supply available P and K in the soil, after consecutive plant harvest (Stamford et al. 2006, 2008, 2011 and 2016). However, phosphate and potassium rocks do not have nitrogen, and therefore do not add to the soil the necessary N for the adequate development of non-leguminous

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plants. The biological nitrogen fixation (BNF) is a complex and dynamic process that is performed by symbiotic, associative, and free-living microorganisms that contribute to increment or reduce the N nutrient in the organic systems (Reis et al., 2006). The increase of N content in the ecosystems or in organic substrates by the performance of diazotrophic bacteria reflects a gain of N higher than the found in environments without the presence of these microorganisms.

The increment of total N in organic materials depends on the inoculation with selected diazotrophic bacteria, and besides this aspect, the bacteria improve the physical and chemical conditions of these materials (Silva et al. 2011, Kowalski et al. 2006). The use of mixed biofertilizers aims to increase nutrient content in the soil, besides increasing productivity and fruit quality (Oliveira et al., 2014). In this sense, the use of organic materials inoculated with free-living diazotrophic bacteria mixed with rock biofertilizers may be a viable and economical alternative in agricultural systems to replace soluble mineral fertilization (Stamford et al. 2017).

Therefore, fertilization with NPK is one of the most important factors, since it affects the production, nutrition and availability of nutrients in the soil, and it is necessary to intensify the use of new techniques in order to increase productivity and to maximize the agricultural system (Stamford et al. 2017; Oliveira et al. 2015, 2014).

These aspects justify the production of a biofertilizer with PK rock biofertilizer mixed with organic material (earthworm compost and sugarcane mud cake) applied in different proportions (3:2:1 and 2:3:1) enriched in N by inoculation with the selected isolates of free-living diazotrophic bacteria, compared with the diazotrophic strain (NFB 2001) actually used to produce the biofertilizer.

2. Material and Methods

2.1 Selection of the diazotrophic bacteria isolated from Brazilian soils

The experiment realized during March to August 2015, in the Laboratory of Environmental Biotechnology at the Department of Agronomy, University Federal Rural of Pernambuco-UFRPE, Brazil, used plastic trays (6 dm^3). The organic matter treatments (5 dm^3) of sugarcane mud cake (MC) and earthworm compost (EC) mixed with rock biofertilizers (BPK) in different proportions (MC:EC:BPK) (3:2:1 and 2:3:1), were inoculated with the six selected effective diazotrophic bacteria. The analyses of the materials used to produce the biofertilizer showed: PK rock biofertilizer - pH=3.5, available P = 14.8 g kg^{-1} and available K = 4.8 g kg^{-1}; Earthworm compost pH= 7.9, total N 8.6 g kg^{-1}, total C= 154 g kg^{-1}, available P = 0.12 g kg^{-1}, available K= 0.13 g kg^{-1} and the sugarcane mud cake pH= 6.9, total N= 8.0 g kg^{-1}, available P= 0.15 g kg^{-1}, available K= 0.5 g kg^{-1}.

Table 1. Soil chemical analyses of the soils used to obtain the diazotrophic bacteria selected to present the best nitrogen fixation (NRA) evaluated by flame chromatography.

Soil	pH (H_2O)	P	Al^{3+}	Ca^{2+}	Mg^{2+}	K^+	Na^+
		mg dm^{-3}	----------	cmol$_c$ dm^{-3}	----------		
S1	5.48	30	0.25	2.80	1.5	0.15	0.31
S2	5.75	53	0.05	3.45	2.3	0.37	0.38
S3	6.63	116	0.00	2.20	1.6	0.40	0.44
S4	5.87	95	0.10	2.05	1.2	0.15	0.38
S5	4.99	38	0.40	1.45	0.9	0.18	0.34
S6	4.98	31	0.48	0.75	0.7	0.26	0.46
S7	5.47	23	0.15	1.15	0.7	0.06	0.25
S8	6.72	192	0.00	2.65	1.8	0.73	0.48

The experiment was set up in a factorial 6x2x2, conducted in a complete randomized design, using the two proportions of organic matters (3:2:1 and 2:3:1), inoculated with six selected effective diazotrophic bacteria, applied in two concentrations (100 and 200 mL), with four replicates. The characteristics of the Brazilian soils used to obtain the selected isolates of diazotrophic bacteria that showed the higher relative nitrogenase activity (RNA) are present in table 1. The soil samples collected in depth 0-20cm were prepared in accordance with Stamford et al. (2007), and then transferred 5 dm^3 to each pot (6 dm^3).

The diazotrophic bacteria were isolated in accordance with Döbereiner et al. (1995), distributing uniformly 200-250mg of soil, in each silica gel plaque, incubated at ± 28 ° C, during 15 days, and observed the colony growth daily. After the bacterial growth, the isolates were transferred to plaque with solid LG medium to obtain pure colonies.

The isolates of diazotrophic bacteria were selected using the results of relative nitrogenase activity (ARA) analyzed by flame ionization chromatography (Perkin Elmer Model F11), following the systematic described in details by Boddey and Döbereiner (1992). The results (table 2), were used to select the best isolates of diazotrophic bacteria obtained from the soils 2, 4, 6 and 7, respectively. The obtained isolates and the two strains used actually to produce the mixed biofertilizers (NFB 1001 e NFB 1003), were grown in LG medium and submitted to analyzes of gene sequence (16S rRNA) processed by the Macrogen Inc. Company (South Korea).

Table 2. Characterization and localities of the soils of the Brazilian Northeast used to obtain the best isolates of the diazotrophic bacteria selected by relative nitrogenase activity (RNA).

Soil reference	Crop system	Local
S1	Area cultivated with mimosa tree legume	Itambé/PE
S2	Area cultivated with Leucaena tree legume	Itambé/PE
S3	Area cultivated with sugarcane crop	Piauí/PI
S4	Area cultivated with sugarcane crop	São Miguel/AL
S5	Area cultivated with sugarcane crop	Ribeirão/PE
S6	Area covered with caatinga system	Garanhuns/PE
S7	Area cultivated with sugarcane crop	Sirinhaem/PE
S8	Area cultivated with mimosa tree legume	Belo Jardim/PE

2.2 Production of the mixed biofertilizers with the organic matters and rocks

The respective treatments were processed after the inoculation with the different effective free-living bacteria, selected previously by evaluation of the relative nitrogenase activity (Lima et al., 2010) and further submitted to gene sequence characterization. The pre-inoculums of the diazotrophic bacteria were initially prepared in Erlenmeyer's of 250 mL, containing 100mL of LG medium, incubating for two weeks under orbital agitation (500 gpm). The material was transferred to Erlenmeyer's of 2000mL with 1000mL of LG medium, to produce the liquid inoculum, incubated for 7 days at orbital agitation (500 gpm), and each tray received 100 mL and 200 mL. The trays with the different treatments incubated at room temperature (28 ± 2 ºC), and the humidity was processed to reach the maximum capacity of water retention, by daily addition of tap water passed throw activated carbon filter.

The experiment was set up in a factorial 6x2x2, with six diazotrophic bacteria applied in two rates, to produce two different biofertilizers using proportions (3:2:1 and 2:3:1), with

four replicates. During the experiment samples collected in various incubation times (0; 10. 20.30 and 40 days) for chemical analyzes and determined pH (H_2O), total C and N, available P and K (Embrapa, 2011).
The data of the treatments in the different incubation times were used to process the statistical analyzes by the SAS Institute software. The means of the treatments were compared by the Tukey test ($p<0.05$).

3. Results and Discussion

3.1 Gene sequence rRNA 16S of the selected diazotrophic bacteria

The gene sequences for rRNA 16S of the selected diazotrophic bacteria isolated from Brazilian soils are shown in table 3.

Table 3. Gene sequence rRNA 16S of the selected diazotrophic bacteria isolates obtained from different Brazilian soils and strains used to produce the mixed biofertilizer.

Strain/Isolates	Description	Similar sequence	Cover (%)	Identity (%)	
Strain NFB 1001	CP 001016.1	*Beijerinckia indica subsp. indica ATCC 9039, complete genome*	73	99	7714
Strain NFB 1003	CP 011534.1	*Bacillus subtilis strain UD1022, complete genome*	55	99	27042
Isolate NFB 4	AJ 295079.1	*Mesorhizobium plurifarium 16S rRNA gene, strain ORS1096 (LMG 15298), complete genome*	92	99	2553
Isolate NFB 6	NR 044403.1	*Paenibacillus castaneae strain Ch-32 16S ribosomal RNA gene, complete genome*	96	96	2468

The diazotrophic bacteria showed high similarity (99%) compared with respective strains of the (National center for Biotechnology Information, 2013). Strain NFB 1003 was similar to *Bacillus subtilis* with the cover of 55%, showing that probably is the same species. The effect of cowpea and leucaena tree legume inoculated with specific rhizobia strain and with *Bacillus subtilis* by Araujo et al. (1999) evaluating the nitrogenase activity, nodulation and N accumulation in plants, and the authors reported increase on N uptake by application of soluble N fertilizer and by the BNF process.
The results of the complete gene sequence also confirm high similarity of 99% for the strain NFB 1001 with *Beijerinckia indica subsp. indica*, and the isolates NFB 04 and NFB 06 with *Mesorhizobium plurifarium* and *Paenibacillus castaneae,* respectively. Valverde et al. (2008) isolated *Paenibacillus castaneae* sp. nov from *Castanea sativa* Miller, based in the phylogenetic analyzes of rRNA 16S gene sequence, reported that this microorganism belongs to the subgroup *Paenibacillus xinjiangensis* and *Paenibacillus glycanilyticus*, with similarity 96.3% and 96.8%, respectively. Thus, similar behavior of the strain in the present study was observed in relation to the level of similarity. Similar results were verified by Costa et al., (2013), that reported similarity of 99% by three strains that nodulate cowpea (strains UFPI BR B3-7; UFPI BR B4-3; UFPI BR B7-6), and suggested that these strains belong to the *Paenibacillus* gene compared with the GenBank data.
Beijerinckia indica subsp. Indica is a strain of the Order *Rhizobiales* that are commonly founded as free-living bacteria in acidic soils and in the rhizosphere and sometimes in the plant phylosphera (Kennedy et al. 2005). These bacteria may be used to promote plant growth and they have great potential to be used in biotechnological studies because can produce

polysaccharides (Scamparini et al., 1997). Most of the genes involved in biological N_2 fixation are clustered in two genomes (10 kb and 51 kb) with the exception of the gene *nif*S which encodes the cysteine desulfurase. Tamas et al., (2010) found lower similarity of these bacteria with sequences of genes deposited in the "GenBank" with 57% for *Beijerinckia indica subsp indica* compared with the gene sequence of *Methylocella silvestris*.
The gene sequence of the gene *nif* could give better information about the bacteria, since; this gene can confirm 100% the diazotrophic character of the isolates. However, there are some limitations regarding the use of the *nif* gene for phylogenetic analysis, depending on the group of microorganisms that is wanted to access (Gaby and Buckley, 2012).

3.2 Analyzes of Nutrients on the Biofertilizers with Diazotrophic

The results for application of diazotrophic bacteria applied in the two concentrations (100 and 200 mL) showed no significant difference for all evaluated parameters, and these data are not present.
The isolates NFB 6 and NFB 2, incubated for 20 days did not affect the pH (Figure 1), with similar values 7.82 and 7.81, respectively. Felix et al., (2014), in lettuce crop observed different results when applied various fertilization treatments (Biofertilizers, Bioprotector and soluble NPK fertilizer) on soil pH do solo, in two consecutive crops.
The evaluation of the pH is of relevant importance, especially in reference to nutrient, because the reaction with acidity or alkalinity of the environment affected substantially the availability of the different nutrients. Thus, the pH effect can increase or reduce the presence of available nutrients in the mixed biofertilizers produced with the different organic matters and may depends of the inoculation of the selected diazotrophic bacteria. Although, increasing the pH the nutrients may be available in a determinate line of values.

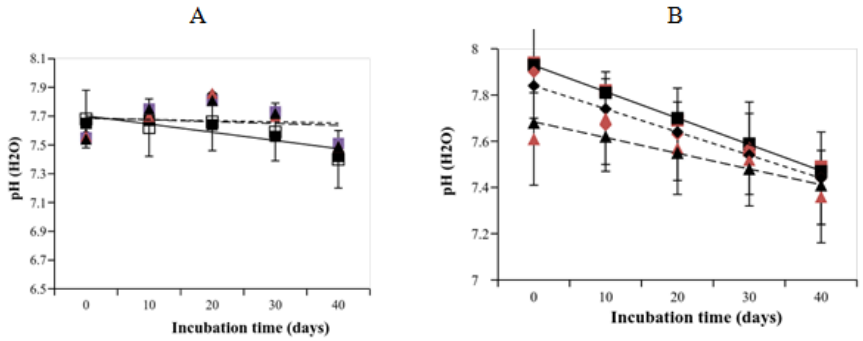

Figure 1 - pH of the mixed biofertilizer with proportion 3:2:1(A) and proportion 2:3:1 (B) inoculated with the different free living diazotrophic bacteria analyzed in different times of incubation. ▲-NBF 6= diazotrophic bacteria isolated from soil 6; ▲-NBF 2= diazotrophic bacteria isolated from soil 2; ■-NBF 1= Strain of diazotrophic bacteria.

The P and K rock biofertilizers inoculated with the *Acidithiobacillus* bacteria displayed very low pH (3.0 to 3.5), in accordance with Stamford et al. (2007), and mixed with organic matters with higher pH the values generally show a significant increase, depending the type and amount of organic matter. The organic matter earthworm compost (pH 7.9) and sugarcane mud cake (7.6) applied in proportions (3:2:1 and 2:3:1)

have values near 7.0 and represent adequate pH to the development of many economic plants.

In reference to the other factor (incubation time), it may be observed interaction and when increase the incubation time determined higher organic matter decomposition and nutrient availability that promote pH reduction, because increasing the organic matter decomposition occur acidity and pH reduction.

The treatments with the diazotrophic bacteria promoted significant interactive effect with the time of incubation and showed increase on total N content (Figure 2).

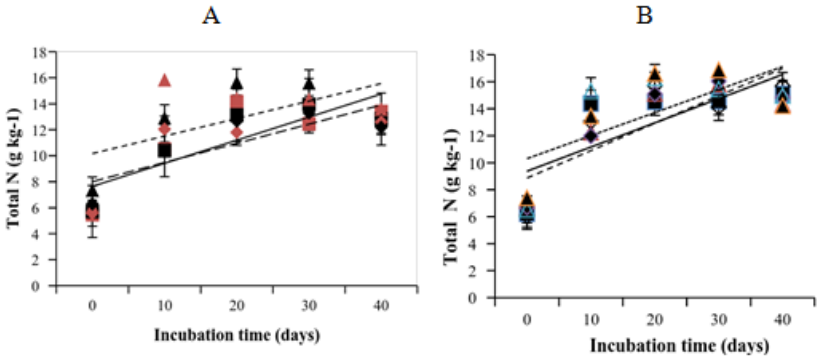

Figure 2 – Total N of the mixed biofertilizer with proportion 3:2:1 (A) and proportion (B) inoculated with the different free-living diazotrophic bacteria analyzed in different times of incubation. ▲-NBF 3= diazotrophic bacteria isolated from soil 3; ▲-NBF 4= diazotrophic bacteria isolated from soil 4; ■-NBF 2= Strain of diazotrophic bacteria isolated and used to produce the biofertilizer.

The strain NFB 1003 influenced positively ($p< 0.05$) increment the total N (17.45 g kg^{-1}) with quadratic behavior in 30 days of incubation. The isolates NFB 2 and NFB 4 showed similar results compared with strain NFB 1003, with reduction of the total N content, and the strain NBF 1003 revealed higher potential to increase total N, probably due to the increment promoted by the process of biological nitrogen fixation.

The results observed in the present work differed from Lima et al. (2010) that studied the effectiveness of different free-living diazotrophic bacteria when inoculated to produce mixed biofertilizer with earthworm compost, and showed increment on total N up to 107% in incubation time of 34 days inoculated with the strain NFB 1001.

In regarding the available P it was observed interaction between the inoculation treatments and the time of incubation that showed a quadratic effect with 2.42, 2.45 and 1.77 1.75 g kg^{-1} with 30 days of incubation when applied the isolates NFB 4, NFB 2 and strain NFB 1001, respectively (Figure 3).

Results with increment of P in organic substrates were reported by Oliveira et al. (2015), evaluating the application of biofertilizers mixed with sugarcane mud cake in proportion 3:1 (MO: BPK), when inoculated with the free-living diazotrophic bacteria NFB 1001. The authors observed a greater increase in available P when applied the mixed biofertilizer compared with soluble fertilizer. Santana et al. (2014) observed similar results, in green pepper grown in field conditions and the increase was proportional with the applied rates.

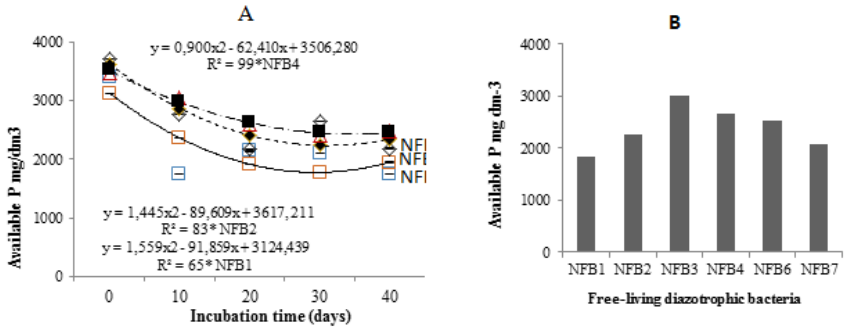

Figure 3 – Available P of the mixed biofertilizer with proportion 3:2:1 (A) and proportion 2:3:1 (B) inoculated with the different free-living diazotrophic bacteria, in different times of incubation. ▲-NBF 4= diazotrophic bacteria isolated from soil 4; ▲-NBF 2= diazotrophic bacteria isolated from soil 2; ■-NBF 1= Strain of diazotrophic bacteria used to produce the biofertilizer.

Probably the reduction in the available P was due to the form of P in the substrate that may be associated with others elements that form products with low availability, depending the environmental conditions. The interaction with others microorganisms as *Acidithiobacillus* and fungi as *Aspergillus* and *Penicillium* may release P from the soil organic matters (Souchie et al. 2007). Some microorganisms have influence in the mineralization/immobilization process and can increase or reduce the nutrients content in the substrates, because the increase in the mineralization process may promote residual effect.

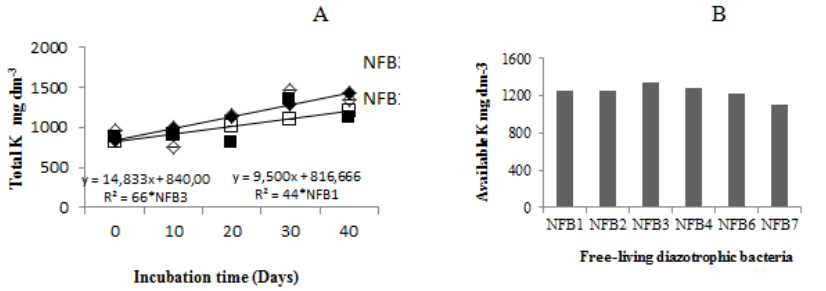

Figure 4 – Available K of the mixed biofertilizer with various incubation time (A) and (B) inoculated with the different free-living diazotrophic bacteria. ▲-NBF 4= diazotrophic bacteria isolated from soil 4; ▲-NBF 2= diazotrophic bacteria isolated from soil 2; ■-NBF 1= Strain of diazotrophic bacteria used to produce the biofertilizer.

The total C did not showed significant interaction ($p>0.05$) and was observed only individual effect of the isolates (Figure 5). The strain NFB 1003 increased the total C (167.8 g kg^{-1}), compared with the others diazotrophic bacteria. The inoculation with strain NFB 1003 probably increased the total N, by the immobilization process and contribute to the reduction of total C in the substrate. In accordance with Kaur et al. (2005), the sugarcane mud cake combined with the mixed biofertilizer promote a significant increase in the total N, P, K and C in the soil. Elsayed et al. (2008) observed similar results.

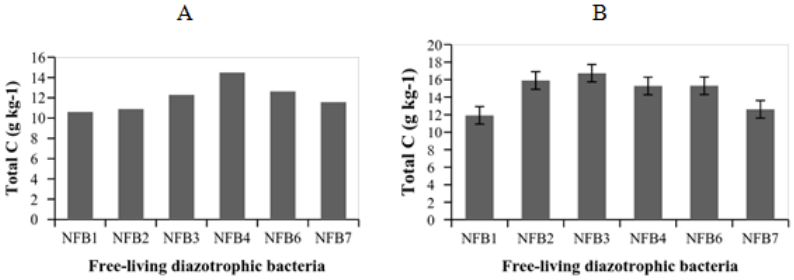

Figure 5 – Total C of the mixed biofertilizer with proportion 3:2:1 (A) and proportion 2:3:1 (B) inoculated with the different free-living diazotrophic bacteria.

Conclusions

The free-living diazotrophic bacteria identified by the 16S rRNA gene sequence showed: strain NFB 1001 = *Beijerinckia indica*, NFB 1003 = *Bacillus subtilis,* Isolate NFB 04 = *Mesorhizobium,* and Isolate NFB 06 = *Paenibacillus*.

The different proportions of organic matters to produce organic biofertilizer contribute to the increment of nutrients to the substrates, and in general the treatments with proportions (3:2:1) and (2:3:1) showed relevant results. The mixed biofertilizers with these organic matters may be alternative to produce economic biofertilizers with potential to replacement of conventional NPK soluble fertilizer.

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