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Microbiological characterization and functional evaluation in formulation made on farm at different times of production

Caracterização microbiológica e avaliação funcional de uma formulação produzida *on farm* em diferentes tempos de produção

Caracterización microbiológica y evaluación funcional en formulación elaborada en granja en diferentes momentos de la producción

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ABSTRACT
Microbial formulations are routinely produced in several rural areas in Brazil. Its production is made using agricultural remnants, easily accessible products and soil microorganisms captured using cooked rice. However, studies on the microbial population and its functions in these products are still very scarce. The aim of this work was to evaluate the microbial diversity and dynamics, its functions and capacity to produce auxins (IAA). Microorganisms were isolated of the formulation with different times of production, quantified by serial dilution and then identified by DNA sequencing. The IAA production was evaluated by high performance liquid chromatography. The results showed a high variability of microorganisms and was extremely variable over production time. The formulation with 10 days of production showed the highest density of microorganisms and IAA concentration. There was no presence of *Salmonella* spp. and the mean number of thermotolerant coliforms is within sanitary limits. In conclusion, the formulation with 10 days of production is the most suitable for use.

Keywords: IAA Production. Microbial Dynamics. Sanitary Control. Alternative Agriculture.

RESUMO
Formulações microbianas são rotineiramente produzidas em várias áreas rurais do Brasil. Sua produção é feita a partir de restos agrícolas, produtos de fácil acesso e microrganismos do solo capturados com arroz cozido. Porém, os estudos sobre a população microbiana desses produtos ainda são muito escassos. Pouco se tem discutido sobre a diversidade microbiana presente nessas formulações, a mudança em sua diversidade ao longo do tempo de produção e metabólitos capazes de auxiliar no crescimento das plantas. O objetivo deste trabalho foi avaliar a dinâmica microbiana, produção de auxinas (IAA). Os microrganismos foram isolados da formulação em diferentes tempos de produção com meios de cultura específicos para cada grupo, quantificados por diluição seriada e posteriormente identificados por sequenciamento de DNA. A produção de IAA foi avaliada por cromatografia líquida de alta eficiência. Análises microbiológicas e parasitológicas (coliformes termotolerantes e Salmonella spp.) Foram realizadas na formulação. Os resultados mostraram

uma alta variabilidade de microrganismos e foram extremamente variáveis ao longo do tempo de produção. A formulação com 10 dias de produção apresentou a maior densidade de microrganismos e concentração de IAA. Não houve presença de Salmonella spp. e o número médio de coliformes termotolerantes está dentro dos limites sanitários. Concluindo, a formulação com 10 dias de produção é a mais adequada para uso.

Palavras-chave: Produção de AIA. Dinâmica Microbiana. Controle Sanitário. Agricultura Alternative.

RESUMEN

Las formulaciones microbianas se producen de forma rutinaria en varias zonas rurales de Brasil. Su producción se realiza utilizando remanentes agrícolas, productos de fácil acceso y microorganismos del suelo capturados utilizando arroz cocido. Sin embargo, los estudios sobre la población microbiana y sus funciones en estos productos son aún muy escasos. El objetivo de este trabajo fue evaluar la diversidad y dinámica microbiana, sus funciones y capacidad de producción de auxinas (IAA). Se aislaron microorganismos de la formulación con diferentes tiempos de producción, se cuantificaron por dilución seriada y posteriormente se identificaron por secuenciación de ADN. La producción de IAA se evaluó mediante cromatografía líquida de alto rendimiento. Los resultados mostraron una alta variabilidad de microorganismos y fue extremadamente variable a lo largo del tiempo de producción. La formulación con 10 días de producción mostró la mayor densidad de microorganismos y concentración de IAA. No hubo presencia de Salmonella spp. y el número medio de coliformes termotolerantes está dentro de los límites sanitarios. En conclusión, la formulación con 10 días de producción es la más adecuada para su uso.

Palabras clave: Producción de IAA. Dinámica Microbiana. Control Sanitario. Agricultura Alternativa.

1 INTRODUCTION

In search of more sustainable alternatives, microbial formulations have been used as alternatives to the use of mineral fertilizers (Alburquerque *et al*., 2012; Owamah *et al*., 2014). These formulations are produced by the farmer, on the rural property, using crop residues, easily accessible materials and microbial inoculants. Different formulations are used, and they differ as to the type of material that is used, the preparation time, the microbial source and the type of digestion that can be aerobic or anaerobic (Owamah *et al*., 2014).

In Brazil, many farmers have been producing microbial formulations on farm in order to produce inoculants sold commercially, as inoculants based on *Bradyrhizobium* spp., *Azospirillum brasilense* e *Bacillus* spp. (Valicente *et al*., 2018) or use bovine manure, litter, poultry litter and soil microorganisms, captured using cooked rice, as a microbial source (Alfa *et al*., 2014) order to inoculate plant growth-promoting microorganisms, which act in important biological processes such as phosphate solubilization, atmospheric nitrogen fixation, plant hormone synthesis and the production of siderophores (Adesemoye *et al*., 2009; Tsai *et al*., 2007). Also, these microorganism are essential for the decomposition of organic matter and the production of secondary elements that are directly linked to plant growth (Magrini *et al*., 2011). However, the fermentation process is usually carried out in unsuitable places, with low aseptic quality, which facilitates contamination and the growth of undesirable microorganisms (Valicente *et al*., 2018). The conditions of production of formulations on farm raise doubts as to the microbiological quality of the product, which may vary as to the ingredients that are used in the formulation, as well as, the time of exposure of the product to the environment.

On farm fermentation leads to the growth of microorganisms such as *Pseudomonas*, *Klebsiella*, *Bacillus*, *Escherichia* and others (Alfa *et al*., 2014; Owamah *et al*., 2014). On farm production is largely carried out in several rural areas in Brazil because they offer the producer a cheap alternative to the use of fertilizers, and mainly, because they believe they are inoculating beneficial microorganisms.

However, little is known regarding the dynamics of the microbial community in this type of formulation due to changes in environmental conditions to which this kind of formulation is submitted. This information can increase knowledge regarding the microorganisms present in these formulations, fluctuations in the diversity of these communities, their ecological role and how temporal distribution occurs, thus contributing to knowledge on the viability of the product in relation to the change of biodiversity. Given the lack of studies on the microbial population present in this type of formulations, the objective of the present study was to characterize the diversity of culturable bacterial (including

actinomycetes) and filamentous fungi and yeasts, detect the presence of possible biological contaminants, and to evaluate the concentration of indole acetic acid (IAA) in different maturation stages of a formulation produced using soil microorganisms as inoculum.

2 MATERIAL AND METHODS

2.1 PRODUCTION OF THE FORMULATION

The formulation utilized in this experiment was a non-commercial product, with a free access formulation, called Hortbio. It was prepared in an Organic Agriculture experimental area in Brasília-DF (geographical coordinate 15º56'61.8S/48º08'42.7O, altitude 997.6 m).

A total of 100 L of the formulation were prepared using blood meal (1.1 kg), rice bran (4.4 kg), castor bean meal (1.1 kg), bone meal (2.2 kg), crushed seeds (1.1 kg), wood ash (1.1 kg), crushed rapadura (0.55 kg) and corn meal (0.55 kg). The microbial inoculum was collected by placing 700 g of rice on a tray covered by a screen, which was buried in an area of dense vegetation (Cerradão) where it remained for seven days. After this period the rice was exposed to a 10% sucrose solution for seven days. The filtrate was used as a source of efficient microorganisms (EM).

The formulation was aerated for a period of 15 minutes every hour. The solution was maintained in plastic bottles with a storage capacity of 200 L, which were sealed to prevent contamination.

Sampling was performed by collecting at different times of production. It were initiated on the day of preparation until 30 days of production, at 5 day intervals, so that the time distribution of sample collection was 0, 5, 10, 15, 20, 25 and 30 days of production. The microbial inoculum used to prepare the formulation was collected for microbiological evaluation. For each sampling time 15 mL were collected in triplicate.

In order to evaluate the dynamics of microorganism populations during the production period, two different formulations were produced. The same

ingredients were used in both, but the first was produced using the EM after activation with sucrose (Bio 0) and the second after 40 days of activation (Bio 40).

2.2 MICROBIOLOGICAL ANALYSES

For each sample time 1 mL of the formulation was homogenized with 9 mL of a 0.85% saline solution. All samples were diluted to 10^{-6} and 100 µL of each dilution were used for plating on different culture media in triplicate.

The Martin medium (Martin, 1949) with streptomycin (100 µg mL^{-1}), the MYGP medium (Masoud *et al*., 2004) with chloramphenicol (100 µg mL^{-1}), the R2A medium (Reasoner; Geldreich, 1985) with cycloheximide (100 µg mL^{-1}), the THSM medium (Williams *et al*., 2003) with chloramphenicol (250 µg mL^{-1}) and streptomycin (100 µg mL^{-1}), and the Starch Casein medium (Kuster; Williams, 1964) with cycloheximide (100 µg mL^{-1}) were used for counting and pure colony isolation of total fungi, yeasts, total bacterial, *Trichoderma* spp. and Actinomycetes, respectively. All Petri dishes were incubated at 25 ºC for five days.

After the microorganism growth period, colony-forming units (CFU mL^{-1}) were counted using the CP600 Plus device (Phoenix Luferco). The results obtained were multiplied by the reciprocal of the dilution used and expressed as CFU mL^{-1}.

Isolation of the microorganisms was achieved by obtaining pure cultures. Bacteria and yeast isolates were stored in a 15% glycerol solution in a freezer at -80 ºC. Filamentous fungi were stored according to the Castellani method (Castellani, 1960).

2.3 MOLECULAR IDENTIFICATION OF MICROORGANISMS

For identification through sequencing only the Bio 0 was used because it presented greater microbial diversity. DNA extraction was conducted from the precipitate of cells grown in liquid medium for five days under stirring. The protocol of Zhu *et al*. (2006) was applied, where SDS, Proteinase K, together with phenol/chloroform is used for bacterial DNA extraction. Yeast DNA was extracted

based on the modified protocol of Kurtzman; Fell (2006), using extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA pH 8, 0.5% SDS). The DNA of filamentous fungi was extracted from cultures grown directly on the Petri dish with culture medium. Extraction was performed by maceration of the mycelium using liquid nitrogen, with application of Cetyl trimethylammonium bromide (CTAB) and phenol chloroform (Michiels *et al*. 2003). For all isolates, the DNA was resuspended in ultrapure water and stored at -20 ºC. Microbial DNA was amplified using the SimpliAmp™ ThermalCycler (Applied Biosystems Inc, Carlsbad, CA, EUA), utilizing the previously extracted DNA samples.

The 16S region of the bacterial ribosomal DNA was amplified using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') as described by Weisburg *et al*. (1991). The PCR reaction was performed for a final volume of 25 µL, containing 1 µM dNTPs (dATP, dTTP, dCTP, dGTP), 0.5 µM of each primer, 1.5 µM $MgCl_2$ and 2.5 U DNA polymerase. The thermocycling program consisted of initial denaturation at 95 ºC for 3 minutes, followed by 30 denaturation cycles at 95 ºC for 1 minute, annealing at 55 ºC for 1 minute and extension at 72 ºC for 1 minute. Lastly, the final extension was performed at 72 ºC for 10 minutes

The D1/D2 domain of the major subunit of yeast ribosomal DNA was amplified using the primers NL1 (5'GCATATCAATAAGCGGAGGAAAAG3') and NL4 (5'GGTCCGTGTTTCAAGAGGG 3'), as described by O'Donnel *et al*. (1993). The PCR reaction was performed to a final volume of 25 µl, containing 20 pmol of each primer, 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. The thermocycling program consisted of initial denaturation at 94 ºC for 3 minutes, followed by 33 denaturation cycles at 94 ºC for 1 minute, annealing at 56 ºC for 30 seconds and extension at 72 ºC for 1 minute. Lastly, the final extension was performed at 72 ºC for 6 minutes.

The ITS region present in fungal ribosomal DNA was amplified using the primers ITS 5 (5’ – TCCGTAGGTGAACCTGCGG–3’) and ITS 4 (5’ – TCCTCCGCTTATTGATATGC-3’) as described by White *et al*. (1990). The PCR reaction was performed for a final volume of 25 µl, containing 0.4 mM of each primer, 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. The thermocycling program consisted

of initial denaturation at 95 °C for 5 minutes, followed by 30 denaturation cycles at 95 °C for 30 seconds, annealing at 62 °C for 1 minute and extension at 72 °C for 2 minutes. The final extension was carried out at 72 °C for 5 minutes.

The generated amplicons were treated with Exo-Sap® (Affymetrix, Santa Clara, CA, EUA) for removal of remaining PCR reagents, and were sequenced using the ABI 3130xl Applied Biosystems sequencer. For the sequencing reaction the Sanger method was used (1997). Identification of the isolates was performed by comparison with sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

2.4 QUANTIFICATION OF INDOLE-3-ACETIC ACID (IAA)

The formulation was evaluated for the presence of IAA on days 10, 20 and 30 of production. Samples were prepared and purified using the SPE cartridge (Sep-pack Oasis® MAX Cartridges) as recommended by Tansupo *et al*. (2010) and Szkop and Bielawski (2013), with some modifications.

The concentration of IAA was determined by high performance liquid chromatography (HPLC) on LC-20AT chromatograph (Shimadzu, Kyoto, Japan). The data were processed in the LC Real Time Analyzes software. The chromatographic separation was performed on C18 column (150 x 4.6 mm dimensions, 5 µm particle size Thermo Cientifica, ODS Hypersil) reverse phase using fluorimetric detector (λEX = 280/ λEM = 350 nm).

All samples and solutions used were filtered on membranes 0.22 µm. Solid phase extraction (SPE) assays were performed with Oasis® Max cartridges (6 mL, 150 mg, Waters, Millford, MA, USA). The standard stock solution of IAA was prepared in ethanol at 100 µg mL^{-1}, and then diluted to the calibration curve (1, 2, 6, 8 and 10 µg mL^{-1}). The linear regression equation was significant ($p < 0.0001$) with $r^2 = 0.995$. The limit of detection was 0.06 µg mL^{-1}. The mobile phase was assembled using the ratio of 25:75 (v: v) 1% acetic acid and acetonitrile, final pH 4.00, at a flow rate of 0.8 mL min^{-1}. The injection volume of the samples was 20 µL. The retention time for IAA in the samples was 7.45 min. The temperature was maintained at 30 °C.

2.5 MICROBIOLOGICAL AND PARASITOLOGICAL QUALITY ASSESSMENT

Thermotolerant coliforms (45 °C) analysis was performed using the Most Probable Number (MPN) method APHA (Kornacki; Johnson; 2001). A 25 g sample was added to 225 mL buffered peptone water. Aliquots were then serially diluted in sterile peptone water to a final dilution of 10^3. Lauryl tryptose broth was used for the presumptive test and EC broth for the confirmatory test. *Salmonella* sp. analysis was performed according to FDA's Bacteriological Analytical Manual (BAM) (Andrews *et al*., 2016). A 25 g sample was added to 225 mL lactose broth. After pre-enrichment, samples were enriched in Rappaport-Vassiliadis and tetrathionate broth and subsequently plated onto bismuth sulphite agar, Hektoen enteric agar, and xylose lysine deoxycholate agar. Spontaneous Sedimentation method (Andrews *et al*., 2016) was used to diagnose infective forms, cysts of protozoan and eggs or larvae of helminths, parasites and commensal, in the formulation samples. The samples were washed with distilled water (five times) every 24 h and the pellet obtained was collected and examined in optical microscope (Olympus BX41) with a 10x and 40x objective lenses.

2.6 STATISTICAL ANALYSES

In the analysis of microorganism dynamics throughout the preparation time of the formulation, regression equations were adjusted, considering a significance level of 5% for the component variables of the equations obtained. The following models were tested: linear regression ($y = ax + b$); quadratic regression ($y = ax^2 + bx+c$), and cubic regression ($y = ax^3 + bx^2 + cx + d$). Regression adjustments and analyses were obtained using the SAS statistical analysis software (Statistical Analysis System, version 9.4).

3 RESULTS AND DISCUSSION

3.1 INFLUENCE OF THE PRODUCTION TIME ON THE DYNAMICS OF MICROBIAL COMMUNITIES AND PRODUCT VIABILITY.

Variation in the number of colonies associated with each group of microorganisms was described by polynomial regressions adjusted for each case (Table 1). Microorganisms belonging to the Fungi Kingdom (including Fungi, yeast and *Trichoderma* in Table 1) showed similar behavior. In Bio 0, produced using EM immediately after activation with sucrose, temporal variation was fit to quadratic polynomial regressions, while in Bio 40 (40 days after EM's activation) temporal variation was fit to linear polynomial regressions.

For Bio 0, the adjusted quadratic regressions to describe the behavior of the fungi population always presented a second derivative greater than zero, which indicates the existence of a minimum point and a concave-up curve. This indicates that there is an initial decrease in the population of these microorganisms followed by an increase, a behavior probably associated with adaptation to the new environment after inoculation of the formulation with the EM. Linear regressions were descriptive of the behavior of these same microorganisms in Bio 40, with EM produced with 40 days. These linear regressions show a behavior of steady increase over time, up to 30 days. Therefore, this maturation was apparently sufficient to avoid the occurrence of the initial microbial population decrease phase associated with the Fungi, as verified for the Bio 0.

Table 1. Explanatory models of the behavior of the different groups of microorganisms isolated from the biofertilizer at different production times

Group of microorganisms	Biofertilizer	Model	Parameters of the equation				
			R^2	β_0	β_1	β_2	β_3
Fungi	Bio 0	$y = \beta_0 + \beta_1 x + \beta_2 x^2$	0.79	5.00 **	-0.07 **	0.003	-
	Bio 40	$y = \beta_0 + \beta_1 x$	0.64	4.17 **	0.06 **	-	-
Yeasts	Bio 0	$y = \beta_0 + \beta_1 x + \beta_2 x^2$	0.73	4.96 **	-0.05 **	0.002**	-
	Bio 40	$y = \beta_0 + \beta_1 x$	0.70	4.12 **	0.07 **	-	-
Trichoderma spp.	Bio 0	$y = \beta_0 + \beta_1 x + \beta_2 x^2$	0.54	4.87 **	-0.06 **	0.003**	-
	Bio 40	$y = \beta_0 + \beta_1 x$	0.70	4.07 **	0.06 **	-	-
Bacteria	Bio 0	$y = \beta_0 + \beta_1 x + \beta_2 x^2$	0.54	6.88 **	0.11 *	-0.006 **	-
	Bio 40	$y = \beta_0 + \beta_1 x + \beta_2 x^2$	0.72	5.43 **	0.24 **	-0.009 **	-
Actinomycetes	Bio 0	$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3$	0.55	5.96 **	0.28 *	-0.03 **	0.0009 **
	Bio 40	$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3$	0.91	4.98 **	0.37 **	-0.03 **	0.0005 **

Bio 0- biofertilizer produced with the EM right after activation; Bio 40- biofertilizer produced with EM after 40 days of activation. *Parameter significant at 5%; **Parameter significant at 1%.
Source: Bonfim (2016)

By analyzing the adjusted equations for Bio 0, it was possible to verify that the minimum points (x_v and y_v) for the populations of filamentous fungi, yeast and *Trichoderma* spp. are 11.66 and 4.59, 12.5 and 4.65, and 10 and 4.57, respectively. Thus, it was observed that the times required to reach the minimum population of these three microorganism groups were estimated at 11.66, 12.5 and 10 days, respectively.

For bacteria, the best fit to the data was obtained using quadratic polynomial regressions for both Bio 0 and Bio 40. Contrary to the quadratic regressions adjusted to the microbial population associated with the filamentous fungi, the curves adjusted to the bacteria presented second derivative less than zero, indicating the existence of a maximum point and a concave-down curve. Therefore, there was an initial increase in the bacterial population, which after reaching its maximum presented a decreasing behavior. The maximum points (x_v and y_v) observed for the bacterial population were, respectively, 9.2 and 7.38; 13.3 and 7.03 for Bio 0 and Bio 40. Thus, it was possible to estimate that the maximum bacteria population in Bio 0 should be reached at 9.2 days, while in Bio 40 should be reached at 13.3 days. The time intervals are similar to those found of the estimated minimum microbial population for the filamentous fungi, which suggests a possible competition between bacteria and fungi over time in the formulations. It is possible that this competition is linked to the competition for resources and better adaptation to the growth medium.

Data on the population of actinomycetes best fit to cubic polynomial regression curves. This indicates a greater population variation of these microorganisms over time, with more frequent fluctuations between maxima and minima than those observed for populations associated with the Fungi and Bacteria kingdom. Therefore, patterns as well-defined in the aforementioned populations were not observed.

Throughout the production process, the microbiota was extremely variable, indicating that the production time is the main factor affecting the dynamics and directly influences the microbial composition. In fact, formulations made on farm have a very diverse microbial community due to dynamic abiotic conditions. Several variables contribute to changes in ecology and microbial growth, such as nutrient and water availability, aeration and concentration of secondary metabolites (Nannipieri *et al*., 2003; Pezzolla *et al*., 2015).

Microorganisms of the Fungi kingdom showed a behavior influenced by the production time. For Bio 0, the observed behavior is possibly associated with an initial population not adapted to the culture conditions to which it was inserted. Therefore, there is a reduction in the microbial density to the minimum point of the curve. The growth phase, observed between 10 and 30 days, suggests the existence of species that best adapt, and in an uncompetitive environment increase their population density. Corroborating this data, Figure 1 B and C show the presence of only *Pichia* species in the yeast culture medium and *Galactomyces candidum* in the isolation of total fungi after 10 and 15 days. These species were possibly better adapted to the cultivation conditions than the other microorganisms. The linear regression was that which best fit to the behavior of Fungi in Bio 40. This data indicates that there was only the phase of microbial growth throughout the formulation production. The stages of adaptation and permanence of only the best adapted microorganisms possibly occurred during the EM maturation phase.

Bacterial growth presented a behavior inverse to that observed for fungi. There was exponential growth, until around 10 days of production, with subsequent reduction. The bacterial population had a higher diversity than the fungal population, and it was not possible to infer which species were best

adapted to the culture conditions. The high bacterial diversity and the fluctuations of bacterial isolation during the preparation period indicate that the formulation environment may have less impact on the change in diversity, but it influences the abundance of the communities. Thus, although the bacteria remain diverse during the production process, the population density is reduced. This possibly occurs due to abiotic limitations, such as the carbon source and potential presence of secondary metabolites.

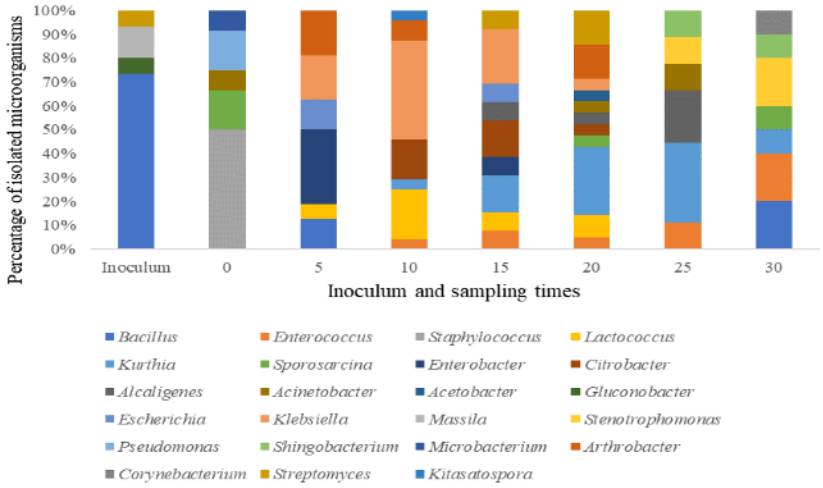
3.2 MICROBIOTA COMPOSITION AND DIVERSITY

A total of two hundred and seventeen microorganisms were isolated and identified in the different preparation times of Bio 0 and EM. Of these, a total of one hundred and twenty bacteria, sixty-one yeasts and thirty-six filamentous fungi were recovered from the formulation in different sample times.

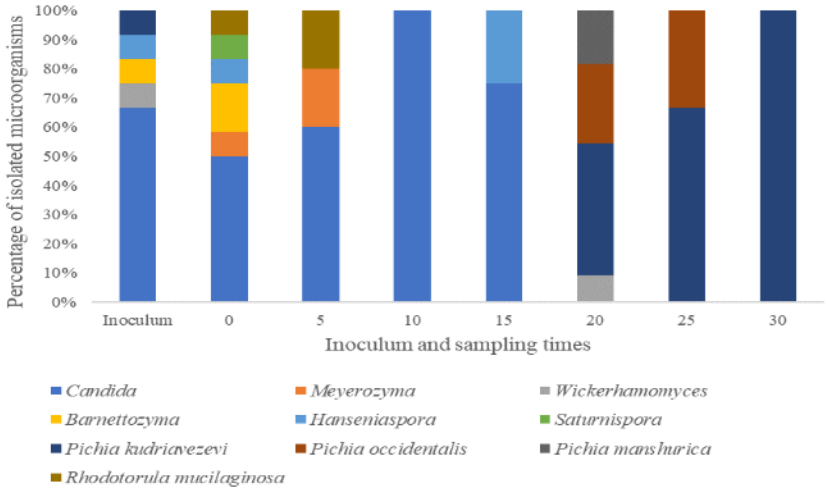
The most abundant bacterial phyla among the isolates were Firmicutes (44%), Proteobacteria (41%), Actinomycetes (13%) and Bacteroidetes (2%). Among the phyla found, 23 bacterial genera were identified: *Bacillus, Enterococcus, Staphylococcus, Lactococcus, Kurthia, Sporosarcina, Enterobacter, Citrobacter, Alcaligenes, Acinetobacter, Acetobacter, Gluconobacter, Escherichia, Klebsiella, Massila, Stenotrophomonas, Pseudomonas, Shingobacterium, Microbacterium, Arthrobacter, Corynebacterium, Streptomyces* and *Kitasatospora.* At 10 days of production the largest number of isolates was recovered, totalizing twenty-one bacteria; at time 0 for preparation of the formulation, eleven isolates, thirteen at time 5, twelve at time 15, fifteen at time 20 and further decreasing at times 25 and 30 with the recovery of nine isolates at each sampling time. In the microbial inoculum fourteen bacteria were isolated. The bacterial genera found in greatest abundance were *Bacillus* spp., *Klebsiella* spp. and *Kurthia* spp. (Figure 1A).

Figure 1. Distribution of microorganisms recovered from the biofertilizer at different sample times and microbial inoculum. A, distribution of bacteria; B, distribution of yeasts; C, distribution of fungi.

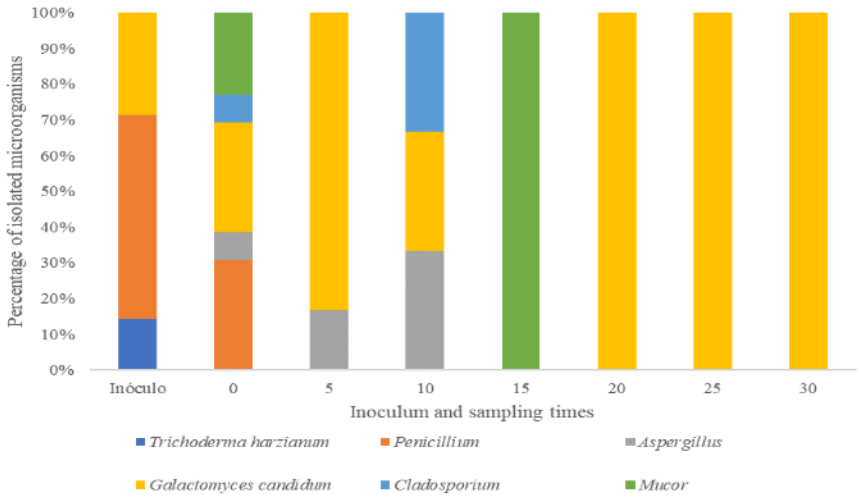
A.



B.



C.



Source: Bonfim (2016)

Among the isolated bacteria, many of those are capable of promote plant growth through biological nitrogen fixation and production of plant stimulators (Avis *et al*., 2008; Janarthine; Eganathan, 2012; Sivasakthi *et al*., 2013; Trabelsi; Mhamdi, 2013).

Members of the family *Enterobacteriaceae*, including bacteria of the genus *Enterobacter*, *Citrobacter*, *Klebsiella* and *Escherichia* were the most abundant in the formulation, isolated at all sample times evaluated. *Enterobacteriaceae* are very abundant in the soil and participate in important biological processes, such as the assimilation of nitrogen (Lin *et al*., 2012). Although many are beneficial and, therefore, their presence is desirable in biological cocktails, the presence of some bacteria of this family in the formulation may indicate a potential source of pathogens and this matter still needs to be clarified, since they are associated with the gastrointestinal tract and are the cause of several human pathologies. Although in the formulation was not detected the presence of *Salmonella* spp., other enteropathogenic bacterial genera were isolated. The presence of enterobacteria was already expected, since they are a bacterial group present in several environments, such as fruits and leaves. However, within this bacterial group, there are species with potential pathogenic to humans and animals, however the results obtained in this study cannot affirm the given pathogenicity related.

A total of sixty-one yeasts were recovered from the formulation. Isolates of the phylum Ascomycota were most prevalent, with predominance of the genera *Candida* and *Pichia*. Only two isolates belonging to the phylum Basidiomycota were recovered, both of the genus *Rhodotorula*. In total, eight different genera were identified: *Pichia*, *Candida*, *Barnettozyma*, *Hanseniaspora*, *Meyerozyma*, *Wickerhamomyces*, *Rhodotorula* and *Saturnispora*. Among the recovered genera, *Candida* and *Pichia* were the most abundant. *Candida* spp. was recovered in the inoculum and at the formulation production times of 0, 5 and 10 days. However, the genus *Pichia* showed predominance only in the last production times, at 20, 25 and 30 days (Figure 1B). The phylogenetic marker used in this study allowed for identification of isolates from the genus *Pichia* at the species level. *Pichia kudriavzevii* was the most abundant species among the recovered *Pichia* isolates. Among the twenty-eight isolates that showed similarity to *Pichia* spp., nineteen

were similar to the species *P. kudriavzevii*. The other identified species of the genus were *P. occidentalis* and *P. manshurica*. *Pichia kudriavzevii* already been isolated from foods, fruits, fermented compounds and the culture of efficient microorganisms (EM). The study of its genome showed the presence of three different genes responsible for the expression of phytase-like proteins, revealing important biotechnological potential of the yeast for production of formulation (Chan *et al*., 2012). Several studies indicate *P. kudriavzevii* as an important phosphate solubilizing yeast (Hellström *et al*., 2012; Greppi *et al*., 2015).

A total of thirty-six filamentous fungi isolates were obtained from the formulation, belonging to the genera: *Galactomyces*, *Penicillium*, *Mucor*, *Aspergillus*, *Cladosporium* and *Trichoderma*. Production time 0 and the inoculum are presented as the times with the greatest number of isolates and greatest diversity. The fungus *Galactomyces candidum* was the most abundant and the only isolate found at the final production times (20, 25 and 30 days) of the formulation (Figure 1C), it was the most resistant to the abiotic conditions existing at these sample times, such as nutritional reduction and possible accumulation of toxins. This species has already been isolated from a wide range of habitats including soil, plant tissues, milk, air, water and the intestinal tract of animals (Pottier *et al*. 2008).

Among the isolated fungi, *Trichoderma harzianum, Penicillium* and *Aspergillus* are indicated as important biocontrol agents, as well as for the promotion of plant growth (Altomare *et al*., 1999; Dal Bello *et al*., 2002; Jeerapong *et al*., 2015). *Trichoderma harzianum* stands out as being the most studied biocontrol agent fungus (Avis *et al*., 2008). In addition to acting as a biocontrol agent, this fungus also contributes with mechanisms related to the promotion of plant growth by the solubilization of soil minerals and organic matter. Altomare *et al*. (1999) showed that *T. harzianum* acts by solubilizing metal oxides such as Zn, Cu, Mn and Fe with high efficiency, a mechanism which may be linked to its biocontrol capacity, making these nutrients unavailable to phytopathogens. It is also possible that the solubilization of metal oxides, commonly found in the mineral matrix of the clay fraction of tropical soils, is capable of releasing phosphate ions specifically adsorbed on these molecules (Fontes; Weed, 1996),

acting to increase P availability. The fungi species *Aspergillus* and *Penicillium* have also been extensively studied with regards to their phosphate solubilization capacity (Khan *et al*., 2009).

Several microorganisms with biotechnological potential and with promising potential to promote plant growth were isolated from the formulation evaluated in this study. Currently in Brazil, on farm production of biological formulations has increased exponentially for use in various crops of agricultural interest. This is the first study that reports microbial diversity in a formulation, with hormonal measurement in the medium of growth of microorganisms and assessment of product quality. There are still doubts as to the microbial quality of these products since they are produced in conditions of low asepsis. Valicente *et al*. (2018) evaluated three formulations produced on farm that used a commercial product containing the bacterium as inoculum *Bacillus thuringiensis*. The analyzed samples, bacteria were identified *Enterococcus casseliflavus* and *Microbacterium* sp., associated with serious infections in humans. Owamah *et al*. (2014) using food scraps and feces as inoculum, isolated several bacteria and fungi with pathogenic potential as *Klebsiella*, *Clostridium*, *Bacillus* e *Pseudomonas*.

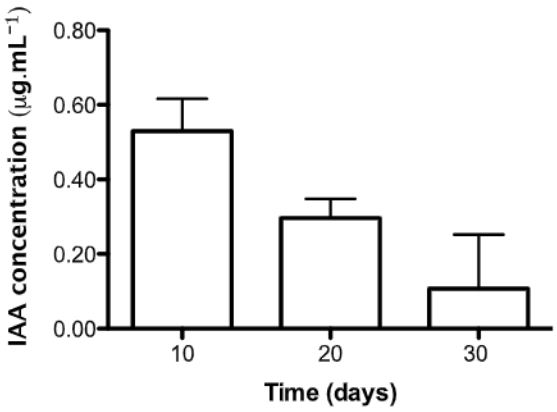
Due to the growth in the use of biological products produced on farm in Brazil, there is a need to pay attention to the quality of these formulations. The results shown in this study show that formulations on farm have a rich microbiota, which has biotechnological potential, however, it does not exclude the possible presence of pathogenic microorganisms.

3.3 QUANTIFICATION OF IAA AT THE DIFFERENT SAMPLE TIMES

The IAA quantification peaks in Bio 0 evaluated after 10, 20 and 30 days of production were well defined and symmetrical for all analyses. The retention time was approximately 9.3 minutes. Concentrations of IAA after 10, 20 and 30 days of fermentation were 0.57, 0.32 and 0.15 µg mL^{-1}, respectively (Figure 2). After 10 days of preparation the formulation presented a concentration equal to 0.57 µg mL^{-1} (Figure 2), a biologically active concentration, necessary to promote the formation of root hair and secondary roots (Zeiger, 2004). The IAA

concentration showed a tendency to decline during the maturation period, with a reduction of 44% between 10 and 20 days of production, and a 74% reduction between days 10 and 30. This fact may be linked to changes in the diversity and abundance of the microorganisms present in the compost and to changes in the composition of the medium, such as the reduction of available nutrients. It is therefore assumed that the microorganisms present at 10 days of formulation production are involved in the production of IAA. The decrease in IAA concentration during the time of production, as well as reduction of the bacterial community suggests that there is a possible relationship between the data. Thus, the time to reach greatest density of the microbial communities, as well as of greatest production of IAA was around 10 days, suggesting that this is the period in which the product is most effective when applied to the soil.

Figure 2. Changes in IAA (Indole-3-acetic acid) concentrations in liquid biofertilizer. The hormone concentration was measured after 10, 20 and 30 days of biofertilizer preparation



Source: Bonfim (2016)

3.4 MICROBIOLOGICAL AND PARASITOLOGICAL QUALITY OF THE FORMULATION

There was no presence of *Salmonella* spp. in any of the evaluated formulations. The mean number of thermotolerant coliforms found was < 11.3 MPN g^{-1}, within the limit imposed by Brazilian legislation (10^3 MPN g^{-1}).

4 CONCLUSION

The results obtained showed that microbial diversity and concentrations of plant growth-promoting molecules, such as Auxin, vary as a function of time. This information is relevant and helps rural producers make decisions about the use of this biofertilizer, which must be used within 10 days after production.

Most of the identified microorganisms are listed in the scientific literature as participants in many important processes for agricultural systems such as phosphate solubilization, siderophore production, nitrogen fixation, biological control, among others. The development and use of bioinputs from these microorganisms is possible and can improve the sustainability of agricultural systems by reducing production costs and reducing negative environmental impacts.

Scientific literature has shown that one of the problems related to the use of biofertilizers and other bioinputs is linked to the great variability of the chemical, physical-chemical and microbiological characteristics of these products. The results of this work can help the development of better standardized bioproducts, guaranteeing more efficient and constant results in productivity and quality in agricultural crops.

The results may also reflect on future research that aims to mitigate greenhouse gas emissions from agricultural systems by reducing the need to use nitrogen fertilizers, as well as those that aim to adapt agriculture to global climate change through the use of bioproducts capable of producing plant growth-promoting molecules such as auxin, which are known to help increase the tolerance of plant species to abiotic stresses such as heat, water deficit, salinization, among others.

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