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Original Article

Thermo-resistant enzyme-producing microorganisms isolated from composting

Microrganismos produtores de enzimas termo-resistentes isolados de compostagem

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Abstract

Organo-mineral fertilizers supplemented with biological additives are an alternative to chemical fertilizers. In this study, thermoresistant microorganisms from composting mass were isolated by two-step procedures. First, samples taken at different time points and temperatures (33 days at 52 ºC, 60 days at 63 ºC, and over 365 days at 26 ºC) were pre-incubated at 80 ºC for 30 minutes. Second, the microbial selection by in vitro culture-based methods and heat shock at 60 ºC and 100 ºC for 2h and 4h. Forty-one isolates were able to grow at 60 °C for 4h; twenty-seven at 100 °C for 2h, and two at 100 °C for 4h. The molecular identification by partial sequencing of the 16S ribosomal gene using universal primers revealed that thirty-five isolates were from eight Bacillus species, one *Brevibacillus borstelensis*, three *Streptomyces thermogriseus*, and two fungi (*Thermomyces lanuginosus* and *T. dupontii*). Data from amylase, phytase, and cellulase activity assays and the enzymatic index (EI) showed that 38 of 41 thermo-resistant isolates produce at least one enzyme. For amylase activity, the highest EI value was observed in *Bacillus licheniformis* (isolate 21C2, EI= 4.11), followed by *Brevibacillus borstelensis* (isolate 6C2, EI= 3.66), *Bacillus cereus* (isolate 18C2, EI= 3.52), and *Bacillus paralicheniformis* (isolate 20C2, EI= 3.34). For phytase, the highest EI values were observed for *Bacillus cereus* (isolate 18C2, EI= 2.30) and *Bacillus licheniformis* (isolate 3C1, EI= 2.15). Concerning cellulose production, *B. altitudinis* (isolate 6C1) was the most efficient (EI= 6.40), followed by three *Bacillus subtilis* (isolates 9C1, 16C2, and 19C2) with EI values of 5.66, 5.84, and 5.88, respectively, and one *B. pumilus* (isolate 27C2, EI= 5.78). The selected microorganisms are potentially useful as a biological additive in organo-mineral fertilizers and other biotechnological processes.

Keywords: thermal-resistant microorganisms, organo-mineral fertilizers, microbial enzymes.

Resumo

Os fertilizantes organo-minerais suplementados com aditivos biológicos são uma alternativa aos adubos químicos. Neste estudo, microrganismos termoresistentes foram isolados de compostagem por procedimentos de duas etapas. Inicialmente, as amostras tomadas em diferentes períodos e temperaturas (33 dias a 52 ºC, 60 dias a 63 ºC e mais de 365 dias a 26 ºC) foram pré-incubadas a 80 ºC por 30 minutos. Posteriormente, a seleção microbiana foi conduzida por métodos baseados em cultura in vitro e choque térmico a 60 ºC e 100 ºC por 2h e 4h. Quarenta e um isolados foram capazes de crescer a 60 °C por 4h; vinte e sete a 100 °C por 2h e dois a 100 °C por 4h. A identificação molecular por sequenciamento parcial do gene ribossomal 16S usando *primers* universais revelou que trinta e cinco isolados eram de oito espécies de Bacillus, um *Brevibacillus borstelensis*, três *Streptomyces thermogriseus* e dois fungos (*Thermomyces lanuginosus* e *T. dupontii*). Os dados dos ensaios de atividade de amilase, fitase e celulase e o índice enzimático (IE) mostraram que 38 dos 41 isolados termorresistentes produziram pelo menos uma enzima. Para a atividade da amilase, o maior valor de IE foi observado em *Bacillus licheniformis* (isolado 21C2, IE = 4,11), seguido por *Brevibacillus borstelensis* (isolado 6C2, IE = 3,66), *Bacillus cereus* (isolado 18C2, IE = 3,52) e *Bacillus paralicheniformis* (isolado 20C2, IE = 3,34). Para a fitase, os maiores valores de IE foram observados para *B. cereus* (isolado 18C2, IE = 2,30) e *B. licheniformis* (isolado 3C1, IE = 2,15). Em relação à produção de celulose, *B. altitudinis* (isolado 6C1) foi o mais eficiente (IE = 6,40), seguido por três *Bacillus subtilis* (isolados 9C1, 16C2 e 19C2) com valores de IE de 5,66, 5,84 e 5,88, respectivamente, e um *B. pumilus* (isolado 27C2, IE = 5,78). Pode-se inferir que os microrganismos selecionados são potencialmente úteis como aditivos biológicos em fertilizantes organo-minerais e outros processos biotecnológicos.

Palavras-chave: microrganismos termoresistentes, Fertlizantes organo-minerais, enzimas microbianas.

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1. Introduction

The agricultural sector exerts a strong influence over the Brazilian economy, contributing positively to the trade balance. In 2019, the sector accounted for 43.2% of the Brazilian exports, which corresponded to US$ 96.79 billion to the balance of trade (ECLAC, 2019). Estimates indicate that by 2024, Brazil will be the largest exporter of food globally (OECD, 2015; Macedo & Nishizaki Júnior, 2017). However, the low availability of nutrients in the Brazilian soils with low amounts of nutrients such as nitrogen (N), phosphorus (P), and potassium (K) is a limiting factor for the development and expansion of agriculture in the country. The low concentrations of available phosphorus in these soils occur due to depletion and run-off carried out over time (Camenzind et al., 2018). Chemical fertilizers are widely used to correct the P deficiency in the soil, but their long-term application is often cumulative, leading to environmental degradation due to eutrophication, which poses a severe threat to water bodies reducing the biodiversity of the aquatic ecosystem (Mastrorilli and Zucaro, 2016; Huang et al., 2017; Jarvie et al., 2018; Bhatt et al., 2019). Also, the high costs of chemical fertilizers is a limiting factor for large-scale food production in Brazil (Martinelli and Filoso, 2009; Mondal et al., 2017; Pivoto et al., 2018). Therefore, due to global restrictions on commodities grown in Brazil the great challenge facing the Brazilian agricultural sector in the near future is to reduce costs and risks by increasing crop productivity in a sustainable way (Pereira et al., 2012; OECD, 2015; Mastrorilli and Zucaro, 2016).

In general, Brazilian soils are impoverished in nutrients necessary for the commercial production of plant foods. The country imports 75% of the fertilizer used to produce food (Galembeck et al., 2019). In the year 2019, the Brazilian fertilizer imports hit a record with the purchases totalizing 31 million tons, spending the US$ 9 billion, and the expectation is that these numbers will continue to increase (Vegro, 2018; BrasilAgro, 2020). Brazil is a country that depends on agriculture, and not producing fertilizer is a significant strategic mistake. The leading fertilizer suppliers to Brazil were Russia, Canada, China, and Morocco, which means that disruption in the supply chain, will lead to significant production problems (Cella and Ross, 2010).

In recent years, a great effort has been devoted to developing and implementing innovative strategies based on eco-friendly products for soil fertilization. The development and implementation of innovative strategies based on eco-friendly products for soil fertilization is a pressing challenge for sustainable agriculture in Brazil since 75% of the fertilizers used in the country are imported (Bhardwaj et al., 2014; Radl et al., 2015; Anda, 2016; Uvarov et al., 2016; Abreu et al., 2017; Correa et al., 2018; Withers et al., 2018). The use of composting technology to produce organo-mineral fertilizers is a low cost and promising solution to ameliorate the environmental pollution problems associated with organic agricultural wastes (Milala et al., 2005; Higashikawa et al., 2010; Shukor et al., 2018). Furthermore, organo-mineral fertilizers have advantages over mineral-derived fertilizers by improving the soil physicochemical properties and even increasing crop yields (Correa et al., 2018). However, one of the limitations of using organo-mineral technology is the increase in temperature of up to 104 ºC during the granulation process that inactivates heat-sensitive microorganisms (Morais, 2014).

Thermophilic microorganisms can grow at elevated temperatures above 45 °C to 70 ºC, with optima between 50 °C and 60 °C, and may be found occupying several habits like hot spring, deep-sea hydrothermal vents, peat bogs, and composting (Hartmann et al., 1989; Panikov et al., 2003; Eze et al., 2011; Zeldes et al., 2015; Counts et al., 2017). In composting, the microbial population degrades organic matter through enzymatic reactions under specific conditions of temperature, moisture, pH, and time, making the compound a stable product (Insam and Bertoldi, 2007; Ribeiro et al., 2017). In the thermophilic phase of composting, the high temperature favors heat-resistant microbial populations (Gou et al., 2017; Xu et al., 2019). This work aimed to select thermophilic enzyme-producer microorganisms from composting mass, aiming their use in the formulation of organo-mineral fertilizers.

2. Material and Methods

2.1. Isolation, counting, and morphological characterization of microorganisms

The samples were collected in triplicate from three rows of organic composting, at three different points in a vegetable production area monitored by the Empresa de Assistência Técnica e Extensão Rural do Estado de Minas Gerais, EMATER-MG.

For isolating the thermo-resistant morphotypes, the collected samples were homogenized, diluted in saline solution (NaCl 0.85%), and dilutions of 10^{-1} were subjected to 80 ºC for 30 minutes under agitation at 120 rotations per minute (Shair, 2014). Then, aliquots of 0.1 mL were transferred to potato dextrose agar (PDA) for bacteria isolation and enumeration using 10^{-4} and 10^{-6} duplicate dilutions (Beever and Bollard, 1970). The fungi isolation was performed in the Martin medium, containing 1mL/L streptomycin at 10^{-2} and 10^{-4} dilutions, in duplicate. For the isolation and counting of actinomycetes, sample dilutions of 10^{-4} and 10^{-6} were plated in duplicate in a specific medium containing L-asparagine, 1 $g.L^{-1}$; glycerol, 10 $g.L^{-1}$; KH_2PO_4, 1 $g.L^{-1}$; plus 100 mg / 100 ml of each of the following micronutrients: $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, and $ZnSO_4.7H_2O$. Then, 15g/L of bacteriological agar and 5mL / L of cycloheximide (0.6% p/ v) solution was added to the medium.

After incubation of the plated samples at 47 ± 1 ºC for three days for bacteria, and five days (120h) for fungi and actinomycetes, the number of bacteria, fungi, and actinomycetes forming colonies were counted, and the result was expressed in Colony-Forming Units (CFU).g^{-1} of the sample. The bacterial colonies were characterized morphologically, according to the Management Manual for the Collection of Multifunctional and Phytopathogenic Microorganisms (CMMF) from the Embrapa Milho e Sorgo

(Paiva et al., 2013). The colonies of bacteria, fungi, and actinomycetes were grouped according to morphology similarities and dissimilarities in an EZ-4 LEICA stereomicroscope. The selected colonies of bacteria and actinomycetes were transferred to Nutrient Agar, incubated at 47 ± 1 ºC, and preserved in mineral oil.

2.2. Evaluation of the thermal tolerance at temperatures above 50 ºC

Pure colonies of bacteria, fungi, and actinomycetes isolated in the previous step were suspended in sterile 0.85% saline and diluted to 10^{-3} and 10^{-5} in Soy Trypticaseine Broth (STB) medium. Then, two replicates of 0.1 mL of each sample were incubated at 60 ºC and 105 ± 5 ºC for 2 h and 4 h. These bacterial colonies were counted by duplicate dilutions of 10^{-2} and 10^{-3}. Bacteria incubated at 105 ± 5 °C were counted by dilution of 10^{-1}. For actinomycetes and fungi, only the presence or absence of growth was annotated.

2.3. Evaluation of enzyme production

The microorganisms showing better heat-resistance to incubation at 60 ºC and 100 ºC for 2 h and 4 h were inoculated, in triplicate, in Petri dishes containing specific solid media for induction of each enzyme. The inoculated plates were incubated in a shaking incubator at 47 ± 1 ºC for 7 days for amylase and phytase, and 10 days for cellulase. After incubation, colonies and halo diameters were measured in centimeters to estimates the Enzyme Index (EI), and expressed by the relationship between the average colony diameter and the average halo diameter. The Starch Agar medium was used for testing amylase production, Phytase production was evidenced in the phytate medium (Pikovskaya, 1948), and for cellulase production, the microorganisms were inoculated in a solid medium supplemented with carboxymethylcellulose (CMC) as the only carbon source (SILVA et al., 2015).

2.4. Statistical analysis

The statistical analysis was performed with the aid of the SISVAR program (Ferreira, 2008). The data were subjected to analysis of variance (ANOVA) with three repetitions and the means compared using the Scott-Knott test at 5% probability level ($p < 0.05$). The estimation of the enzymatic index (EI) was according to a completely randomized design.

2.5. Molecular characterization

2.5.1. Bacterial and actinobacteria 16S rDNA amplification

Genomic DNA of bacterial and actinomycetes was extracted using the Promega Wizard Genomic DNA Purification Kit following the manufacturer's instructions. The 16S universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used in PCR reactions. The reaction consisted of 40 ng of DNA, 1X Promega buffer, 1.5 mM $MgCl_2$, 0.125 mM dNTP, 4% (v/v) dimethyl sulfoxide (DMSO), 0.4 µM of each primer and 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 µL. The amplification conditions were 95 °C for 2 min followed by 35 cycles of 94 °C for 30 sec, 59 °C for 30 sec, and 72 °C for 90 sec, followed by a final elongation step at 72°C for 10 min. The amplicons were checked in agarose gel electrophoresis 1% (w/v) in TAE 1X buffer (Tris-acetate-EDTA), and purified with ExoSAP-IT one-step solution (Applied Biosystems).

2.5.2. PCR amplification of the fungal internal transcribed spacer (ITS)

The extraction of fungal genomic DNA was according to the CTAB method described by Doyle and Doyle (1990). The amplification of the fungal ITS region the universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') were used. The PCR reactions were performed with 20 ng of DNA, 1X buffer, 2.0 mM $MgCl_2$, 0.125 mM dNTP, 0.5 µM of each primer, and 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The amplification conditions were 95 °C for 2 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 sec, and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. Amplicons were resolved by agarose gel electrophoresis 1% (w/v) in TAE 1X buffer (Tris-acetate-EDTA), and purified with ExoSAP-IT one-step solution (Applied Biosystems).

2.5.3. DNA Sequencing

The sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer instructions (Applied Biosystems), and the sequencing run was in an ABI PRISM 3500XL Genetic Analyzer (Applied Biosystems). The nucleotide sequences were aligned and edited by Sequencher 4.1 program, and the species identity determined by the Blastn algorithm located in NCBI (https://www.ncbi.nlm.nih.gov/). The edited sequences were compared with the nucleotide sequences deposited in the GenBank database. The rDNA sequences have been registered in the GenBank database under accession numbers shown in Table 1.

3. Results

3.1. Isolation, counting, and morphological characterization of heat-resistant microorganisms from composting mass

The average temperature of the C1 sample (33th day) was 51.9 ºC, with counts of 4.0 x 10^7 CFU.g^{-1} for bacteria, 2.0 x 10^7 CFU/g^{-1} for actinomycetes, and 1.1 x 10^3 CFU.g^{-1} for fungi. The sample C2 (60th day) showed an average temperature of 63 ± 1 ºC and counts of 5.8 x 10^6 CFU.g^{-1}, 1.8 x 10^6 CFU.g^{-1}, and 1.7 x 10^5 CFU.g^{-1} for bacteria, actinomycetes, and fungi, respectively. The sample C3 (more than 12 months), the temperature was 25 ± 1 ºC, and its microbial community was more sensitive to rising temperature (80 ºC), with a count of 1.0 x 10^5 UFC.g^{-1} of bacteria and absence of fungi and actinomycetes. We succeeded in isolating 105 microorganisms: 85 from bacteria, 20 from actinomycetes, and two fungi.

Table 1. Isolates identification, Colony-Forming Units (CFU), growth temperature, and enzymatic index (EI).

Identification	GenBank acession	Species	CFU	Growth Temperature				Enzymatic Index (EI)		
			Time Zero	60 °C/2h	60 °C/4h	100 °C/2h	100 °C/4h	Amylase	Phytase	Cellulase
BRM043901	MH305321	*Bacillus licheniformis*	3.0 x 10^4	6.4 x 10^4	1.2 x 10^5	4.6 x 10^2	-	4.11	1.63	1.00
BRM043903	MH305322	*Bacillus licheniformis*	1.6 x 10^5	3.5 x 10^4	6.5 x 10^3	1.2 x 10^3	-	3.00	1.13	1.00
BRM043904	MH305323	*Bacillus licheniformis*	7.9 x 10^6	2.9 x 10^4	2.5 x 10^4	4.4 x 10^2	-	1.96	1.69	1.00
BRM043905	MH305325	*Bacillus licheniformis*	3.5 x 10^6	1.8 x 10^5	4.4 x 10^5	3.8 x 10^3	-	1.89	1.35	1.00
BRM043906	MH305324	*Bacillus licheniformis*	3.2 x 10^5	2.0 x 10^4	2.0 x 10^4	7.0x 10^2	3.7 x 10^2	2.01	1.42	1.49
BRM043909	MH305329	*Bacillus licheniformis*	4.9 x 10^6	1.9 x 10^4	1.2 x 10^5	7.8 x 10^2	-	2.04	1.45	0.00
BRM043910	MH305326	*Bacillus licheniformis*	2.7 x 10^5	1.7 x 10^4	1.6 x 10^5	2.4 x 10^2	-	2.10	1.25	1.00
BRM043912	MH305327	*Bacillus licheniformis*	2.6 x 10^7	2.6 x 10^5	9.4 x 10^4	3.3 x 10^2	-	1.23	1.44	1.00
BRM043913	MH305328	*Bacillus licheniformis*	8.0 x 10^6	5.0 x 10^4	2.7 x 10^4	4.7 x 10^3	-	1.92	1.60	1.00
BRM043915	MH305330	*Bacillus licheniformis*	6.4 x 10^5	7.8 x 10^4	3.7 x 10^4	2.9 x 10^2	-	1.63	1.67	1.00
BRM043917	MH305335	*Bacillus licheniformis*	1.2 x 10^7	1.3 x 10^4	1.1 x 10^5	7.0 x 10^2	-	2.90	1.70	1.00
BRM043919	MH305336	*Bacillus licheniformis*	1.6 x 10^7	2.3 x 10^5	2.0 x 10^4	2.1 x 10^3	-	1.10	1.80	1.00
BRM043920	MH305331	*Bacillus licheniformis*	8.0 x 10^5	8.2 x 10^4	5.8 x 10^4	2.2 x 10^2	3.3 x 10^2	2.49	1.16	1.00
BRM043921	MH305332	*Bacillus licheniformis*	2.0 x 10^5	1.2 x 10^3	4.9 x 10^3	>10^6	-	1.40	2.15	1.00
BRM043922	MH305337	*Bacillus licheniformis*	1.4 x 10^7	1.8 x 10^5	5.0 x 10^4	9.0 x 10	-	1.62	1.46	1.00
BRM043923	MH305333	*Bacillus licheniformis*	1.1 x 10^7	4.1 x 10^5	4.2 x 10^5	1.3 x 10^3	-	1.59	1.83	1.00
BRM043924	MH305334	*Bacillus licheniformis*	3.2 x 10^5	1.9 x 10^5	3.5 x 10^4	-	-	2.74	0.18	1.00
BRM043925	MH305338	*Bacillus licheniformis*	5.9 x 10^4	1.2 x 10^4	5.2 x 10^4	-	-	2.10	1.49	1.00
BRM043926	MH305339	*Bacillus licheniformis*	1.3 x 10^5	7.6 x 10^4	9.4 x 10^4	-	-	2.06	1.52	1.00
BRM043927	MH305340	*Bacillus licheniformis*	2.5 x 10^5	4.1 x 10^4	1.6 x 10^4	3.2 x 10^2	-	1.57	1.54	0.00
BRM043964	MK351618	*Bacillus licheniformis*	1.5 x 10^7	3.3 x 10^4	5.8 x 10^4	-	-	0.00	0.00	0.00
BRM043968	MK351619	*Bacillus licheniformis*	1.0 x 10^7	7.8 x 10^4	3.2 x 10^3	2.2 x 10^4	-	0.00	0.00	1.00
BRM043979	MK351620	*Bacillus licheniformis*	1.3 x 10^7	1.7 x 10^5	1.9 x 10^5	4.3 x 10^2	1.7 x 10^2	0.00	0.00	0.00
BRM043907	MH305356	*Bacillus paralicheniformis*	6.0 x 10^6	4.0 x 10^4	2.0 x 10^4	>10 6	-	3.34	1.21	1.20
BRM043908	MH305341	*Bacillus subtilis*	1.2 x 10^7	1.7 x 10^5	2.9 x 10^4	2.0 x 10^3	-	1.61	1.32	5.84
BRM043911	MH305342	*Bacillus subtilis*	1.0 x 10^7	1.8 x 10^4	2.5 x 10^4	2.4 x 10^3	-	1.30	1.57	5.88

*EI = means of three repetitions. The values of enzymatic index (EI) represent the relationship between the average diameter of the degradation zone and the average diameter of the colony.

Table 1. Continued...

Identification	GenBank acession	Species	CFU	Growth Temperature				Enzymatic Index (EI)		
			Time Zero	60 °C/2h	60 °C/4h	100 °C/2h	100 °C/4h	Amylase	Phytase	Cellulase
BRM043914	MH305343	*Bacillus subtilis*	3.9×10^7	2.8×10^4	2.5×10^4	1.9×10^3	-	1.65	1.65	3.17
BRM043918	MH305344	*Bacillus subtilis*	6.7×10^6	7.5×10^4	1.8×10^4	8.0×10^2	-	2.19	0.00	5.66
BRM043928	MH305345	*Bacillus subtilis*	1.7×10^6	5.9×10^4	1.0×10^5	3.2×10^3	-	2.05	1.36	1.00
BRM043900	MH305354	*Bacillus cereus*	1.0×10^7	1.2×10^5	1.3×10^5	-	-	3.52	2.30	0.00
BRM043902	MH305355	*Bacillus pumilus*	4.4×10^6	4.5×10^6	2.4×10^5	4.0×10^3	-	0.00	0.00	5.78
BRM043929	MH305351	*Bacillus megaterium*	3.0×10^5	1.0×10^4	8.2×10^3	-	-	1.80	1.11	0.00
BRM043916	MH305357	*Bacillus altitudinis*	2.0×10^7	1.1×10^5	4.9×10^4	1.9×10^3	-	0.00	0.00	6.40
BRM043935	MH305350	*Bacillus clausii*	3.6×10^4	4.2×10^4	6.4×10^4	-	-	1.71	0.00	0.00
BRM043933	MH305349	*Bacillus clausii*	1.7×10^4	1.4×10^4	2.0×10^3	-	-	1.49	0.00	0.00
BRM043932	MH305353	*Brevibacillus borstelensis*	2.7×10^6	1.5×10^4	4.8×10^4	2.9×10^2	-	3.66	0.00	2.01
BRM043930	MH305346	*Streptomyces thermogriseus*		+	+	-	-	2.05	0.00	1.58
BRM043931	MH305348	*Streptomyces thermogriseus*		+	+	-	-	1.45	0.00	1.63
BRM043934	MH305347	*Streptomyces thermogriseus*		+	+	-	-	2.29	0.00	1.97
BRM043937	MH305320	*Thermomyces lanuginosus*		+	+	-	-	1.24	1.14	0.00
BRM043938	MH305319	*Thermomyces dupontii*		+	+	-	-	1.53	0.00	0.00

*EI = means of three repetitions. The values of enzymatic index (EI) represent the relationship between the average diameter of the degradation zone and the average diameter of the colony.

3.2. Evaluation of heat shock resistance (temperature above 50 ºC)

Of the 85 bacteria tested for heat stress, 75 survived at 60 ºC for two hours, and of these, 72 survived at 60 ºC for four hours. Twenty-eight resisted to 100 ºC for two hours from the last group, and three survived to incubation at 100 ºC for four hours. Among the twenty actinomycetes isolates, eight were resistant to incubation at 60 ºC for two and four hours, and none survived at 100 ºC. The two fungal isolates were unable to grow at 60 ºC or 100 ºC. After the thermal stress, forty-one different morphotypes, 36 of bacteria, three actinomycetes were identified, and together with the two fungal isolates, they were selected for molecular identification.

3.3. Molecular identification

The 16S rDNA sequencing results of the 41 isolates confirmed the morphological identification of the thirty-six bacteria, three actinomycetes, and two fungi. Among the 36 bacterial isolates, 35 belonged to the genus *Bacillus*, with the species *B. licheniformis* predominating (23 isolates), followed by *B. subtilis* (5 isolates), *B. clausii* (2 isolates), and one of each of *B. cereus*, *B. paralicheniformis*, *B. pumilus*, *B. megaterium B. altitudinis*. One bacterial isolate was *Brevibacillus bortolensis*. The three isolates of Actinomycetes were identified as Streptomyces *thermogriseus*, and the two fungi were *Thermomyces lanuginosus* and *T. dupontii*. The isolates with their respective GenBank accession numbers are shown in Table 1.

3.4. Evaluation of enzyme activity

Of the 41 isolates tested for enzyme production, using the criterion for Enzyme Index (EI) values ≥ 2, Seventeen were amylase producers, three phytase, and seven cellulose producers. Of these, two strains BRM043901 and BRM043903 of *Bacillus licheniformis*, and one strain of *B. paralicheniformis* (BRM043907) were heat shock resistant to the treatment at 100 °C/2h, the EI for amylase were equal to 4.11, 3.00, and 3.34, respectively (Table 1). For amylase, two other strains of *B. licheniformis* (BRM043906 and BRM043920), resistant to 100 °C/4h, obtained an EI equal to 2.01 and 2.49, respectively. The highest EI value for phytase was 2.30 for the strain BRM043900 of *B. cereus* resistant to the treatment at 60 °C/4h. For cellulase, the group of isolates resistant to 60 ºC/2h or 60 ºC/4h, and 100 ºC/2h, the highest EI values ≥ 5.0 were observed for *B. subitilis* isolates BRM043908 (EI=5.84) and BRM043911 (EI=5.88), *B. pumilus* BRM043902 (EI=5.66), and *B. altitudinis* BRM043916 (EI=6.40). Two actinomycetes (BRM043930 and BRM043934) showed EI values of 2.05 and 2.29 for amylase production, respectively. The two fungi (BRM043937 and BRM043938) were not efficient in enzymes production with EI values varying from 0.0 to 1.53 (Table 1).

4. Discussion

In our study, the microorganisms were isolated from aerobic organic composting of mixed vegetables waste (co-composting). The composting system is a natural breakdown of dead organic matter made by a continuous succession of microbial communities and small saprophyte organisms (Insam and Bertoldi, 2007; Irvine et al., 2010; Song et al., 2014). The size and complexity of a transient microbial community in the composting vary according to biological, chemical, and physical factors like moisture content, temperature, pH, surface area, particle size, the volume of the composting mass, and aeration (Zakarya et al., 2018; Meng et al., 2019; Piazza et al., 2020). As the organic matter decay is based on exothermic reactions, a natural rise in temperature occurs during the composting process. Thus, the temperature is a critical limiting variable for the evolution of a composting since the succession of the microbial community and their representativeness in the degradation phases are highly dependent on this parameter (Oliveira et al., 2004; Haruta et al., 2005; Fujino et al., 2005; Insam and Bertoldi, 2007; Rebollido et al., 2008; Irvine et al., 2010; Heck et al., 2013; Castro-Fernández et al., 2018). The conventional composting processes typically comprise four major microbiological stages concerning temperature: mesophilic, thermophilic, cooling, and maturation (Rebollido et al., 2008; Irvine et al., 2010). During the process, the microbial community changes, and the final product is the compost (Irvine et al., 2010). When composting temperature exceeds 40 ºC, the thermophilic microorganisms start to predominate in composting and rapidly accelerate the organic matter decomposition (Insam and Bertoldi, 2007; Ribeiro et al., 2017). As the temperature increases above to 65 ºC, most mesophilic microorganisms are eliminated, and those thermoresistant or thermophilic turn predominant. Thus, as the temperature rises and falls, different microbial species become more or less active (Partanen et al., 2010; Neher et al., 2013). When the temperature drops, the most thermophiles die off, and the compost enters into the mature stage. Mesophiles, which has been working in a reduced capacity around the colder area of the decomposing, will become more active (Ansari and Hanief, 2015). In our study, the average temperature of the C1 sample was 51.9 ºC, with counts of 4.0×10^7 CFU.g^{-1} for bacteria, 2.0×10^7 CFU.g^{-1} for actinomycetes, and 1.1×10^3 CFU.g^{-1} for fungi. The sample C2 showed an average temperature of 63 ºC and counts of 5.8×10^6 CFU.g^{-1}, 1.8×10^6 CFU.g^{-1}, and 1.7×10^5 CFU.g^{-1} for bacteria, actinomycetes, and fungi, respectively. In the sample C3, after more than 12 months of the initial composting process, the temperature was 25 ± 1 ºC. We were interested primarily in isolating thermophilic microorganisms; thus, the samples were collected at the thermophilic stage (C1 at 51 ± 1 ºC, and C2 at 63 ± 1 ºC) and maturation (C3 at 25 ± 1 ºC). In these two stages, the composting mass was colonized at a significant proportion by bacteria (79.4%), followed by actinomycetes (18.7%) and in lower numbers by fungi (1.9%).

The molecular characterization of the thermoresistant isolates showed that 88% (36 isolates) were *Bacillus* spp., and in this group, 64% were *B. licheniformis*. The other major group was *B. subtilis* (14%) with five isolates. This result confirms previous studies describing that *Bacillus* spp. is the primary group of microorganisms frequently isolated from different compost masses (Souza and Martins, 2001; Zainudin et al., 2013; López-González et al., 2014;

López-González, et al., 2015; Chin et al., 2017; Ribeiro et al., 2017). The high frequency of thermophilic *Bacillus* spp. in composting may be associated with its ability to form resistant spores under environmental conditions that kill most other organisms. Thus, the ability to form spore confer an adaptive advantage to *Bacillus*, and several strains from composting produce hydrolytic enzymes, biosurfactants, antibiotics, and plant growth promoters (Yakimov et al., 1995; Dhanarajan et al., 2017; Jeong et al., 2017; Hsu et al., 2018; Sonune and Garode, 2018). Some thermophilic bacteria show a high metabolic capacity to degrade recalcitrant substances such as lignin, which is particularly interesting for industrial applications (Charbonneau et al., 2012; Gautam et al., 2012; Bhattacharya and Pletschke, 2014; Gonzalo et al., 2016; Ahirwar et al., 2017; Siu-Rodas et al., 2018).

Although all composting has many common characteristics, such as mesophilic, thermophilic, and maturity phases, other peculiar attributes are unique (Partanen et al., 2010). Microbial groups are frequently detected in moderate or high counts at all or many composting stages (Ribeiro et al., 2017). Galitskaya et al. (2017), using the pyrosequencing technique to study the fungal and bacterial successions in co-composting of organic wastes, found that the number of 16S bacterial copies (~10^6) were superior to fungi ITS (~10^5) in the initial stage of the process. In the present study, five species were isolated at all sampling times, and 36 out of 41 were Firmicutes. Whereas some species were present at almost every sampling time, i.e., *Bacillus licheniformis*, most isolates were found at low frequency or, in one or two samples (actinomyces and fungi).

B. licheniformis has excellent potential for practical and biotechnological processes. The sequencing data of the complete genome of *B. licheniformis* revealed 82 genes encoding hydrolytic enzymes for accelerating the composting hydrolysis process polysaccharides, proteins, lipids, and other compounds for biotechnological uses (Rey et al., 2004). The comparison between *B. licheniformis* and *B. subitilis* genomes identified 27 extracellular proteins in *B. licheniformis* that were absent in *B. subitilis* (Rey et al., 2004). Also, in the fungi group, Janusz et al. (2017) found that different wood degradation strategies possibly correlate to the number of genes coded for secretory enzymes. Several strains of thermoresistant *Bacillus licheniformis* isolated from composting produce hydrolytic enzymes, biosurfactant compounds for environmental decontamination, antimicrobial activity, and plant growth (Yakimov et al., 1995; Dhanarajan et al., 2017; Hsu et al., 2018; Sonune and Garode, 2018). Thermophilic strains of *B. licheniformis* produce a soluble biopolymer that enhances the mobilization and solubilization of oil contaminants in the soil (Dhanarajan et al., 2017). Hsu et al. (2018) used the probiotic properties of a *B. licheniformis* strain to remove up to 70% of the mycotoxin zearalenone contamination in cereal grains. Benzoic acid from *Bacillus licheniformis* strain MH48 was useful for controlling various fungal plant pathogens (Jeong et al., 2017).

At the end of the thermophilic stage, the bacterial and fungal species composition changed significantly, while their relative abundance decreased. During the later composting stages, the dominant bacterial and fungal communities remained active, but their relative abundance decreased. In our study, the bacterial community members were very stable and isolated at high frequency in all treatments, while the frequency of fungi was very low and isolated only in the thermophilic stage. These results show that the dynamic of microbial communities in composting may vary due to the high complexity of many biological and environmental parameters, such as the initial microbial population, temperature, pH, and moisture interacting in a specific way (Chandna et al., 2013). According to the study by Villar et al. (2016), the nature of the starting material determined the microbial dynamics in composting systems. In our study, the dynamics of the microbial community in the composting were not similar. In our study, the microbial communities underwent significant changes from the first to second thermophilic phase (C1 to C2) during the 33rd to the 60th-day interval. The bacterial counting gradually decreases over the three stages (10^7, 10^6, and 10^5), while actinomycetes and fungi increased during the second thermophilic phase and became countless after the composting maturation. In a recent study, Galitskaya et al. (2017) found that although the dominating taxa of bacterial and fungal communities remained during the later composting stages, their relative abundance decreased.

Biologically active enzymes have been obtained from plants, animals, and microorganisms. However, high amounts of microbial enzymes stable at various extreme conditions are easily isolated in fast, low-cost production and show high reliability for industrial processes and applications (Gopinath et al., 2017). Microbial enzymes involved in the composting process as cellulases, amylase, β-glucosidases, proteases, ureases, phytase, phosphatases, and arylsulphatases are potential candidates for biotechnological applications. These enzymes are active in cellulose depolymerization, hydrolysis of amide and glycosidic bonds, nitrogen mineralization, and release of phosphate and sulfate groups from organic compounds (Bernardi et al., 2018). In this study, we tested three enzymes production by microorganisms isolated from organic composting: amylase, phytase, and cellulase.

Amylases are necessary enzymes employed in the food industry to hydrolyze starch molecules into polymers composed of glucose units (Berry and Paterson, 1990). The α-amylase is the most critical enzyme to initiate the starch hydrolysis reaction (Windish and Mhatre, 1965). Microbial amylases have been primarily used for the starch breakdown in the industrial sectors. Baratto et al. (2011) observed that α-amylase from fungal origin decrease their activity rapidly at temperatures above 50 ºC, while in bacterial, the enzyme has high stability to elevated temperatures. In a study, Wu et al. (2018) demonstrated the thermo resistance of an acidophilic α-amylase produced by *B. licheniformis* isolated from hot springs (Wu et al., 2018). In our study, seventeen isolates among B. *licheniformis*, *B. subtilis*, *B. cereus*, *Bacillus paralicheniformis*, *Brevibacillus borstelensis*, and *Streptomyces thermogriseus* are promising for use in the food and starch processing industries.

Phytate (inositol hexaphosphate), in the salt form of phytic acid, represents the primary storage form

of phosphorus (P) and inositol in many plant tissues (Cosgrove, 1980; Ravindran et al., 1994). Phytate strongly interacts with divalent minerals in the soil, such as magnesium, calcium, zinc, and iron, and becomes highly unavailable as a P source for microorganisms, plants, and animals (George et al., 2007; Menezes-Blackburn et al., 2016). Therefore, phytate is considered an anti-nutritive compound for non-ruminant animals since they lack the enzyme phytase necessary to solubilize the stored P in the phytate compound (Dersjant-Li et al., 2015). Hence, the undigested phytate negatively affects the absorption of minerals and other nutrients, inhibiting certain digestive enzymes, and binding to carbohydrates and vital proteins required for energy and growth (Hallberg et al., 1989; Greger, 1999; Bohn et al., 2004; Phillippy, 2006; Selle et al., 2000; Amina et al., 2017). For this reason, several food processing and preparation methods use the enzyme phytase to reduce the phytate content of cereals and legumes (Jatuwong et al., 2020). Microbial phytase has been considered the most promising enzyme in food processing and feed pellet manufacturing industries specialized in birds and non-ruminant animals (Dersjant-Li et al., 2015; Amina et al., 2017). However, phytase and other enzymes are sensitive to high temperatures, 80 ºC or more, applied during the pelleting process. Thermostable phytase may be a suitable candidate for feed processing and supplements (Konietzny and Greiner, 2004). Thus, identifying novel thermotolerant phytase isoforms can be of significance in food and feed processing industries.

It has been shown that phytate-utilizing bacteria may improve P-bioavailability in composting and P acquisition by plants (Richardson et al., 2005; Fuentes et al., 2006). Therefore, studies on composting microbial community and its phytate degradation ability might contribute to ameliorate the compost quality and provide new strains for biotechnological processes. Microbial inoculants have been studied as a strategic tool to enhance the Pi-availability in organic wastes and plants. Menezes-Blackburn et al. (2016) demonstrated that Pi-availability in cattle manure was increased by inoculating phytase-producing bacteria in the composting of agricultural wastes. The authors concluded that phytase-producing bacteria inoculation represents an attractive strategy to increase Pi-availability in agricultural wastes with potential applications as organic fertilizers in crops and pastures (Menezes-Blackburn et al., 2016). Microbial phytase activity in soil has been more frequently detected in fungi followed by bacteria, and most of the soil bacteria detected to possess phytase activity belong to the Gammaproteobacterial group (Amina et al., 2017). In our study, only two bacterial strains from Firmicutes showed EI ≥ 2.0. The result, together with the fact that none of the fungi isolates was efficient in phytase production, highlights that thermotolerant phytase-efficient microorganisms at the time of implementing future composting systems may improve the quality of the final compost.

Cellulose, hemicelluloses, and lignin are essential components of the primary plant cell wall and the most abundant organic polymer on Earth. Cellulose has a broad use in industry to produce paper and various derivative products such as cellophane, rayon, fibers, and other miscellaneous products. Cellulose contains large reservoirs of energy that provide real potential for conversion into biofuels. Conversion of cellulose into biofuel, such as cellulosic ethanol, is under development as a renewable fuel since 1898 (Harris et al., 1945; Mosier et al., 2005). Cellulase is an important enzyme involved in the composting process. Cellulase works at temperatures typically above 50 °C with the temperature optima at 70 °C. Mayende et al. (2006) isolated thermophilic bacteria from organic composting capable of producing thermophilic cellulases at 60 ºC and 70 ºC and extremely thermophilic at 80 ºC with potential for application in industrial biotechnology. Rocha (2010) isolated a thermoresistant strain of *Bacillus cereus* from hot springs in the Brazilian state of Goiás, and the selected strain is grown up at 50 ºC and showed high cellulolytic activity. In the present study, *Bacillus subtilis, Bacillus pumilus*, and *Bacillus altitudinis* were high producers of cellulase with EI values above to 5.0., thus they have a high potential application in biotechnological processes.

5. Conclusions

The present study's main contribution is the isolation and characterization of new thermophilic and thermotolerant isolates from composting that may be tested as inoculant activators to accelerate the composting processes in organo-mineral fertilizer production; and applications in many biotechnological processes requiring high temperatures. The bacterial group was more resistant to thermal stress than fungi and actinomycetes, and more efficient in producing the hydrolytic enzymes amylase, phytase, and cellulase. Spore-forming bacteria from firmicutes are the most frequent microbial group in composting mass. Thermoresistant *B. licheniformis* strains, resistant to treatment at 100 ºC for 4h, were more efficient in producing amylase. The thermoresistant *Bacillus stratosphericus* (100 ºC for 2h) showed the highest EI value for cellulase (EI= 6.4). The present study's main contribution is the isolation and characterization of new thermophilic and thermotolerant isolates from composting that may be tested as inoculant activators to accelerate the composting processes in organo-mineral fertilizer production; and applications in many biotechnological processes requiring high temperatures.

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