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Endophytic bacteria isolated from *Phaseolus vulgaris* produce phytases with potential for biotechnology application

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Abstract. Currently, endophytic microorganisms have become a good source of different enzymes and others metabolites of industrial interest. Among a huge spectral of molecules, enzymes as phytases have been emphasized by the ability to hydrolyze the phytic acid that represents the largest storage form of inorganic phosphorus in cereals, which are the staple diet of monogastric animals such as swine and poultry. Moreover, phytic acid acts as an antinutrient by chelating divalent metal ions, and it is interesting provide phytase as an animal feed supplement for those monogastric animals. In the current study, 158 endophytic bacteria isolated from the leaves of three cultivars of *Phaseolus vulgaris* were assessed for the ability to produce phytase. Among them, four isolates belonging to the *Pseudomonas*, *Stenotrophomonas*, *Microbacterium* and *Rhodococcus* genera were highlighted, due their phytase production. The phytase produced by *Microbacterium foliorum* BAC1157 exhibited activity at 70 °C and stability in the presence of divalent cations, indicating that this phytase has a promising use in the animal feed industry. To the authors' knowledge, this is the first report on phytase production by bacteria of the *Microbacterium* genera.

Keywords: Endophytic bacteria; *Microbacterium*; Bean; Phytase activity.

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Introduction

Endophytes are microorganisms that can be isolated from surface-disinfested healthy plant tissue and do not harm the host plant (Hallmann et al., 1997). These microorganisms are considered potential sources of compounds such as antibiotics, antioxidants, anticancer drugs and enzymes (Strobel and Daisy, 2003). Phytase activity were detected in the endophytic bacteria belonging the genera *Bacillus*, *Streptomyces*, *Acinetobacter*, and *Rhizobium* isolated from the seeds and roots of *Phaseolus vulgaris* (common bean) (López-López et al., 2010). The phytases, or myo-inositol hexakisphosphate phosphohydrolases (E.C. 3.1.3.8; 3.1.3.26; 3.1.3.72), are derived from various sources, including animals, plants and microorganisms (Konietzny and Greiner, 2002; Vohra and Satyanarayana, 2003). These enzymes are notable for being able to hydrolyze phytate making available the inorganic phosphorus (Pi) and phosphorylated *myo*-inositols (Ariza et al., 2013). Phytic acid (myo-inositol hexakisphosphate) and the mixture of the cationic salts of phytic acid, which is commonly known as phytate, are organic phosphorus compounds that are widespread in nature. Approximately 60% to 90% of the Pi in vegetables, oilseeds and cereals is stored in the form of phytic acid (Coelho et al., 2002; Rao et al., 2009).

Monogastric animals such as swine and poultry are unable to degrade the phytic acid in food (Schroder et al., 1996). Therefore, the diet of these animals should be supplemented with Pi to meet their nutritional needs. However, approximately 70% of the Pi supplemented in diets is excreted into the environment, triggering the eutrophication of bodies of water near farm areas (Turner and Haygarth, 2000). Furthermore, phytic acid acts as an antinutritional factor by chelating divalent cations (such as Zn^{2+}, $Fe^{2+/3+}$, Ca^{2+}, Mg^{2+}, Mn^{2+} and Cu^{2+}), proteins, starch and lipids, thus preventing their absorption throughout the gastrointestinal tract (Urbano et al., 2000). Thus, the importance to add feed enzymes on animal diet is that it can have an effect on the microbiome of the gastrointestinal tract promoting gut absorption of Pi and other cations for the animals (Kiarie et al. 2013; Tortola et al., 2013).

Most of the microbial phytases described in the literature are acidic phytases, with an optimal pH and temperature range of 4.0 to 5.5 and 45 °C to 60 °C, respectively (Lei et al., 2003; Mullaney and Ullah, 2003; Patki et al., 2015). The optimal temperature for most microbial phytases is approximately 50 °C (Simon and Igbasan, 2002); however, some microbial phytases have exhibited optimal temperatures above 60 °C (Berka et al., 1998; Matsui et al., 2000; Zamudio et al., 2001). These enzymes can dephosphorylate phytate into several position isomers of myo-inositol pentakis-, tetrakis-, tris-, bis- and monophosphates (Greiner et al., 2001). Several myo-inositol isomers show pharmacological antitumor and antiangiogenic (Maffucci et al., 2005), and anti-inflammatory (Claxson et al., 1990) effects, in addition to preventing diabetes complications (Carrington et al., 1993). For these and other reasons, phytases have tremendous potential in biotechnology, especially human and animal nutrition and bioremediation.

The global market for feed enzyme has growing in the next years; it has valued at $899.19 Million in 2014 and is estimated to reach $1,371.03 Million by 2020 according to market research firm - MarketsandMarkets (http://www.marketsandmarkets.com). Among the major enzymes applied, phytases correspond for 60% to 80% of the market for animal nutrition (Corrêa et al., 2015). Thus, endophytic microorganisms that are promising sources of phytases have been evaluated. Accordingly, the aim of this study was to assess the phytases produced by the

endophytic bacteria isolated from three varieties of the common bean, *Phaseolus vulgaris* (Costa et al., 2012), and to characterize the enzymes from these promising bacterial isolates.

Matherials and methods

One hundred fifty-eight strains of endophytic bacteria were isolated from the leaves of the Talismã, Ouro Negro and Vermelhinho cultivars of the common bean (Costa et al., 2012) and used for screening phytase production. The bacterial isolates were activated in 10 mL of 10% TSA culture media (Araujo et al., 2002) at 28 °C and 150 rpm for 48 to 72 h. Each bacterial isolate was inoculated into the phytase screening media plates described by Kim et al. (2003) Phytase production was assessed by the formation of a hydrolysis halo around the colonies 48 and 96 h after incubation. Bacterial isolates showing larger hydrolysis halos were selected for further testing. The strains selected were activated in TSA media as previously described to quantify phytase activity. An aliquot of the cell culture was used to inoculate 50 mL of phytase screening media (PSM), which consisted of 1.5 g L^{-1} glucose, 0.5 g L^{-1} NH_4NO_3, 0.05 g L^{-1} KCl, 0.05 g L^{-1} $MgSO_4.7H_2O$, 0.001 g L^{-1} $FeSO_4.7H_2O$, 0.001 g L^{-1} $MnSO_4.7H_2O$ and 5.00 g L^{-1} phytic acid calcium salt ($C_6H_{16}CaO_{24}P_6$- P9539-Sigma®) at a pH of 5.5. The cultures were maintained at 28 °C and 150 rpm for 48 h. After this period, the broth was centrifuged at 3,000 g for 5 min, and the supernatant was used to determine the enzymatic activity.

In order to characterize the enzyme produced by the endophytic isolates, the phytase activity was determined by incubating 50 µL of supernatant in 350 µL of 0.1 M sodium acetate buffer, pH 5.0, with 2.5 mM phytic acid sodium salt ($C_6H_{18}O_{24}P_6$ xNa^+ yH_2O- P0109-Sigma®) at 50 °C for 15 min. The Pi released was quantified using the method described by Heinonen and Lahti (1981). A standard curve with several concentrations of KH_2PO_4 was used to calculate the enzymatic activity. One unit of phytase activity was defined as the amount of enzyme required to release 1 µmol of phosphate per minute under the assay conditions. The effect of temperature on phytase activity was measured by incubating the culture filtrate with the substrate at temperatures ranging from 20 °C to 80 °C for 15 min. The effect of pH on enzyme activity was assessed by incubating the culture supernatant and the substrate in the following (0.1 M) buffer solutions: glycine-HCl (pH 1.0-3.5), sodium acetate (pH 4.0-6.0), Tris-HCl (pH 6.5-8.5) and glycine-NaOH (pH 9.0-12.0). The enzyme-substrate specificity was determined by replacing the phytic acid sodium salt with 2.5 mM ATP, ADP, pyrophosphate or β-glycerophosphate. The effect of divalent cations on phytase activity was determined by incubating the supernatant of each bacterial isolate with 1 mM, 5 mM, 10 mM or 20 mM $CaCl_2$, $CdSO_4$, $CuSO_4$, $FeSO_4$, KCl, $MgCl_2$, $MnCl_2$ or $ZnSO_4$ for 1 h. The 500 mM stock solution of each cation was prepared as described in Tang et al. (2006). The untreated enzyme activity was set to 100% in all of the assays. The data were analyzed using analysis of variance and Tukey's test at 5% probability using SAEG software, version 9.1, Universidade Federal de Viçosa.

The 158 bacterial isolates were inoculated in solid media with calcium phytate to qualitatively assess phytase production. Among them, 45 (28.48%) exhibited a hydrolysis halo around the colonies 48 h and 96 h after inoculation (Table 1). The most promising isolates with hydrolysis halos were *Microbacterium foliorum* BAC1157, *Pseudomonas aeruginosa* BAC3115, *Rhodococcus erythropolis* BAC2162 and *Stenotrophomonas maltophilia* BAC2135, with 15.5 cm (48 h), 22.0 cm (96 h), 15.0 cm (48 h) and 22.0 cm (96 h) hydrolysis halos, respectively. The bacterial isolates were subsequently cultured in liquid

induction media to quantify phytase production (Figure 1). Of these cultures, *R. erythropolis* BAC2162 and *S. maltophilia* BAC2135 showed the highest phytase activities (0.659 and 0.625 U mL^{-1}, respectively), while *M. foliorum* BAC1157 showed the lowest activity (0.550 U mL^{-1}).

Table 1. The *in vitro* phytase production assay from the 158 endophytic isolates of the *P. vulgaris* leaf and hydrolysis halo size of the 45 isolates positive for phytase production.

Isolate	Number of access	Phytase production	Halo size (cm)	
			48 h	96 h
Bacillus thuringiensis strain BAC1001	HM355592	-	0	0
Micrococcus luteus strain BAC1002	HM355593	-	0	0
Microbacterium testaceum strain BAC1003	HM355594	-	0	0
Micrococcus luteus strain BAC1004	HM355595	+	14.5	17
Micrococcus luteus strain BAC1005	HM355596	-	0	0
Microbacterium testaceum strain BAC1006	HM355597	+	12.5	14.5
Enterobacter asburiae strain BAC1007	HM355598	+	13	15
Microbacterium testaceum strain BAC1008	HM355599	-	0	0
Microbacterium testaceum strain BAC1009	HM355600	+	11	15
Enterobacter hormaechei strain BAC1010	HM355601	+	12	15
Rhizobium larrymoorei strain BAC1011	HM355602	-	0	0
Stenotrophomonas maltophilia strain BAC1012	HM355603	+	14	17,5
Microbacterium testaceum strain BAC1013	HM355604	+	14	17
Microbacterium testaceum strain BAC1014	HM355605	+	12	15
Bacillus niacini strain BAC1015	HM355606	-	0	0
Staphylococcus epidermidis strain BAC1016	HM355607	-	0	0
Bacillus thuringiensis strain BAC1017	HM355608	+	12.5	15.5
Microbacterium testaceum strain BAC1018	HM355609	-	0	0
Microbacterium testaceum strain BAC1019	HM355610	-	0	0
Microbacterium testaceum strain BAC1020	HM355611	-	0	0
Rhizobium larrymoorei strain BAC2021	HM355612	+	12	16
Microbacterium testaceum strain BAC2022	HM355613	-	0	0
Methylobacterium populi strain BAC2023	HM355614	-	0	0
Stenotrophomonas maltophilia strain BAC2024	HM355615	-	0	0
Rhizobium larrymoorei strain BAC2025	HM355616	-	0	0
Staphylococcus epidermidis strain BAC2026	HM355617	+	9.5	16
Brevibacillus agri strain BAC2027	HM355618	+	13	16
Methylobacterium populi strain BAC2028	HM355619	+	12.5	17.5
Microbacterium testaceum strain BAC2029	HM355620	-	0	0
Staphylococcus warneri strain BAC2030	HM355621	+	10	16
Stenotrophomonas maltophilia strain BAC2031	HM355622	-	0	0
Staphylococcus warneri strain BAC2032	HM355623	-	0	0
Methylobacterium populi strain BAC2033	HM355624	+	7	12.5
Staphylococcus epidermidis strain BAC2034	HM355625	-	0	0
Staphylococcus epidermidis strain BAC2035	HM355626	-	0	0
Microbacterium testaceum strain BAC2036	HM355627	+	0	4
Sphingomonas dokdonensis strain BAC2037	HM355628	-	0	0
Sporosarcina aquimarina strain BAC2038	HM355629	-	0	0
Staphylococcus caprae strain BAC2039	HM355630	-	0	0
Methylobacterium populi strain BAC2040	HM355631	-	0	0
Staphylococcus epidermidis strain BAC3041	HM355632	-	0	0

Table 1. Continued.

Isolate	Number of access	Phytase production	Halo size (cm)	
			48 h	96 h
Bacillus thuringiensis strain BAC3042	HM355633	-	0	0
Microbacterium foliorum strain BAC3043	HM355634	+	12	15.5
Bacillus bataviensis strain BAC3044	HM355635	-	0	0
Sphingobacterium multivorum strain BAC3045	HM355636	+	11.5	14.5
Staphylococcus epidermidis strain BAC3046	HM355637	-	0	0
Microbacterium testaceum strain BAC3047	HM355638	-	0	0
Bacillus amyloliquefaciens strain BAC3048	HM355639	-	0	0
Staphylococcus kloosii strain BAC3049	HM355640	-	0	0
Microbacterium phyllosphaerae strain BAC3050	HM355641	-	0	0
Methylobacterium populi strain BAC3051	HM355642	-	0	0
Stenotrophomonas maltophilia strain BAC3052	HM355643	-	0	0
Bacillus bataviensis strain BAC3053	HM355644	-	0	0
Methylobacterium populi strain BAC3054	HM355645	-	0	0
Staphylococcus warneri strain BAC3055	HM355646	-	0	0
Sporosarcina sp. strain BAC3056	HM355647	-	0	0
Acinetobacter sp. strain BAC3057	HM355648	-	0	0
Acinetobacter radioresistens strain BAC3058	HM355649	+	14.5	16
Lysinibacillus sphaericus strain BAC3059	HM355650	-	0	0
Microbacterium testaceum strain BAC1061	HM355651	-	0	0
Staphylococcus epidermidis strain BAC1062	HM355652	-	0	0
Bacillus thuringiensis strain BAC1063	HM355653	-	0	0
Methylobacterium populi strain BAC1064	HM355654	+	14	18.5
Microbacterium testaceum strain BAC1065	HM355655	-	0	0
Microbacterium testaceum strain BAC1066	HM355656	-	0	0
Microbacterium foliorum strain BAC1067	HM355657	-	0	0
Methylobacterium populi strain BAC1068	HM355658	-	0	0
Methylobacterium populi strain BAC1069	HM355659	-	0	0
Brevundimonas vesicularis strain BAC1070	HM355660	-	0	0
Staphylococcus epidermidis strain BAC2071	HM355661	-	0	0
Frigoribacterium faeni strain BAC2072	HM355662	-	0	0
Sphingomonas sanguinis strain BAC2073	HM355663	-	0	0
Staphylococcus warneri strain BAC2074	HM355664	-	0	0
Frigoribacterium faeni strain BAC2075	HM355665	-	0	0
Rhizobium larrymoorei strain BAC2076	HM355666	-	0	0
Staphylococcus epidermidis strain BAC2077	HM355667	-	0	0
Bacillus subtilis strain BAC2078	HM355668	-	0	0
Microbacterium testaceum strain BAC2079	HM355669	-	0	0
Microbacterium testaceum strain BAC2080	HM355670	-	0	0
Brevibacillus agri strain BAC3081	HM355671	-	0	0
Methylobacterium populi strain BAC3082	HM355672	-	0	0
Methylobacterium populi strain BAC3083	HM355673	-	0	0
Paenibacillus cineris strain BAC3084	HM355674	-	0	0
Staphylococcus epidermidis strain BAC3085	HM355675	-	0	0
Microbacterium foliorum strain BAC3087	HM355676	-	0	0
Methylobacterium populi strain BAC3088	HM355677	-	0	0
Staphylococcus warneri strain BAC3089	HM355678	-	0	0
Methylobacterium populi strain BAC3090	HM355679	-	0	0
Paenibacillus cineris strain BAC1091	HM355680	+	0	8
Microbacterium testaceum strain BAC1092	HM355681	-	0	0
Microbacterium testaceum strain BAC1093	HM355682	-	0	0
Microbacterium phyllosphaerae strain BAC1094	HM355683	-	0	0

Table 1. Continued.

Isolate	Number of access	Phytase production	Halo size (cm)	
			48 h	96 h
Micrococcus luteus strain BAC1095	HM355684	-	0	0
Bacillus muralis strain BAC1096	HM355685	-	0	0
Microbacterium sp. strain BAC1097	HM355686	-	0	0
Kocuria palustris strain BAC1098	HM355687	-	0	0
Microbacterium testaceum strain BAC1099	HM355688	-	0	0
Microbacterium testaceum strain BAC1100	HM355689	-	0	0
Staphylococcus saprophyticus strain BAC2101	HM355690	-	0	0
Staphylococcus warneri strain BAC2102	HM355691	-	0	0
Methylobacterium populi strain BAC2103	HM355692	-	0	0
Delftia tsuruhatensis strain BAC2104	HM355693	-	0	0
Methylobacterium populi strain BAC2105	HM355694	-	0	0
Bacillus sp. strain BAC2106	HM355695	-	0	0
Staphylococcus warneri strain BAC2107	HM355696	-	0	0
Methylobacterium populi strain BAC2108	HM355697	-	0	0
Staphylococcus warneri strain BAC2109	HM355698	-	0	0
Frigoribacterium faeni strain BAC2110	HM355699	-	0	0
Pseudomonas aeruginosa strain BAC3111	HM355700	+	13	17.5
Staphylococcus warneri strain BAC3112	HM355701	+	12.5	14.5
Methylobacterium populi strain BAC3113	HM355702	-	0	0
Dietzia cinnamea strain BAC3114	HM355703	-	0	0
Pseudomonas aeruginosa strain BAC3115	HM355704	+	15.5†	19.5
Staphylococcus warneri strain BAC3116	HM355705	-	0	0
Agromyces mediolanus strain BAC3117	HM355706	-	0	0
Agromyces mediolanus strain BAC3118	HM355707	-	0	0
Staphylococcus warneri strain BAC3119	HM355708	-	0	0
Stenotrophomonas sp. strain BAC3120	HM355709	-	0	0
Stenotrophomonas maltophilia strain BAC3121	HM355710	-	0	0
Stenotrophomonas maltophilia strain BAC3122	HM355711	-	0	0
Stenotrophomonas maltophilia strain BAC3123	HM355712	-	0	0
Agromyces mediolanus strain BAC3124	HM355713	-	0	0
Stenotrophomonas maltophilia strain BAC3125	HM355714	-	0	0
Microbacterium testaceum strain BAC2126	HM355715	+	14.5	20.5
Stenotrophomonas maltophilia strain BAC2127	HM355716	-	0	0
Stenotrophomonas maltophilia strain BAC2128	HM355717	+	12.5	16
Agromyces mediolanus strain BAC2129	HM355718	-	0	0
Agromyces sp. strain BAC2130	HM355719	-	0	0
Staphylococcus epidermidis strain BAC2131	HM355720	-	0	0
Methylobacterium populi strain BAC2132	HM355721	-	0	0
Paenibacillus lautus strain BAC2133	HM355722	-	0	0
Stenotrophomonas maltophilia strain BAC2134	HM355723	+	10.5	15
Stenotrophomonas maltophilia strain BAC2135	HM355724	+	14.5	22.0†
Stenotrophomonas maltophilia strain BAC1136	HM355725	+	10	15
Pseudomonas aeruginosa strain BAC1137	HM355726	+	13	16.5
Enterobacter asburiae strain BAC1138	HM355727	+	12.5	15
Enterobacter hormaechei strain BAC1139	HM355728	+	13.5	16.5
Bacillus thuringiensis strain BAC1140	HM355729	+	13.5	16.5
Bacillus thuringiensis strain BAC1141	HM355730	+	15	21
Rhizobium larrymoorei strain BAC2142	HM355731	+	14	16
Staphylococcus warneri strain BAC2143	HM355732	-	0	0
Staphylococcus sp. strain BAC2144	HM355733	+	13	16

Table 1. Continued.

Isolate	Number of access	Phytase production	Halo size (cm)	
			48 h	96 h
Paenibacillus sp. strain BAC2145	HM355734	+	14	16
Bacillus bataviensis strain BAC2147	HM355735	-	0	0
Stenotrophomonas maltophilia strain BAC3148	HM355736	-	0	0
Stenotrophomonas maltophilia strain BAC3149	HM355737	+	10.5	16.5
Stenotrophomonas maltophilia strain BAC3150	HM355738	-	0	0
Bacillus thuringiensis strain BAC3151	HM355739	-	0	0
Staphylococcus warneri strain BAC1152	HM355740	-	0	0
Microbacterium testaceum strain BAC2153	HM355741	+	11.5	18
Microbacterium testaceum strain BAC3154	HM355742	-	0	0
Stenotrophomonas maltophilia strain BAC3155	HM355743	-	12	15
Delftia tsuruhatensis strain BAC1156	HM355744	-	0	0
Microbacterium foliorum strain BAC1157	HM355745	+	15.0†	17
Delftia tsuruhatensis strain BAC2158	HM355746	+	12.5	15
Delftia tsuruhatensis strain BAC3159	HM355747	+	11	13
Stenotrophomonas maltophilia strain BAC2160	HM355748	+	13	18
Rhodococcus erythropolis strain BAC2162	HM355749	+	14.5	22.0†

† Isolates selected for phytase activity evaluation

The phytase produced by the endophytic isolates was characterized for optimal activity temperature and pH, substrate specificity and stability in the presence of divalent cations. *M. foliorum* BAC1157 contained the phytase with the highest optimal temperature (70 °C), followed by *R. erythropolis* BAC2162 and *S. maltophilia* BAC2135 (50 °C) and *P. aeruginosa* BAC3115 (40 °C) (Figure 2). The pH studies revealed that *M. foliorum* BAC1157, *P. aeruginosa* BAC3115 and *R. erythropolis* BAC2162 exhibit two peaks of maximum phytase activity (pH 3.0 and 5.0, 3.0 and 8.0 and 4.5 and 8.0, respectively), while *S. maltophilia* BAC2135 showed a single peak of activity at pH 4.5 (Figure 3).

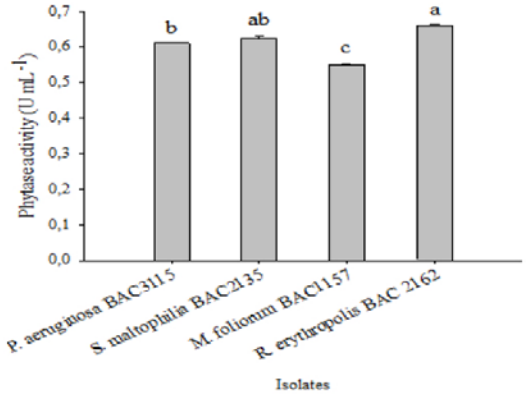

Figure1. Activity of the endophytic isolate-derived phytases. The enzyme activity was measured in phytase-inducing liquid media after 48 hours of culture. 1 - *Pseudomonas aeruginosa* strain BAC3115; 2 - *Stenotrophomonas maltophilia* strain BAC2135; 3 - *Microbacterium foliorum* strain BAC1157; 4 - *Rhodococcus erythropolis* strain BAC2162. The bars followed by the same letter in the same column do not differ in Tukey's test at 5% probability.

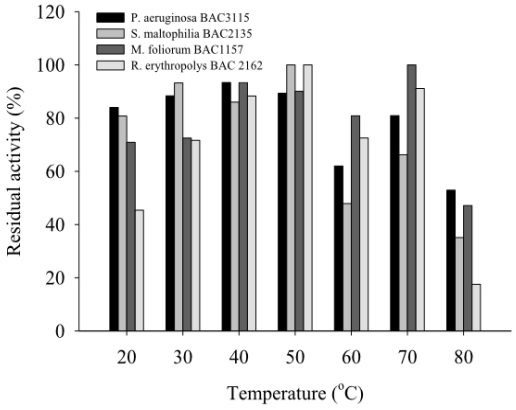

Figure 2. Optimal temperatures for the phytases derived from the four endophytic isolates. 115 - *Pseudomonas aeruginosa* strain BAC3115; 135 - *Stenotrophomonas maltophilia* strain BAC2135; 3 - 157 *Microbacterium foliorum* strain BAC1157; 162 - *Rhodococcus erythropolis* strain BAC2162.The optimal temperatures were determined in phytase-inducing liquid media after 48 hours of culture.

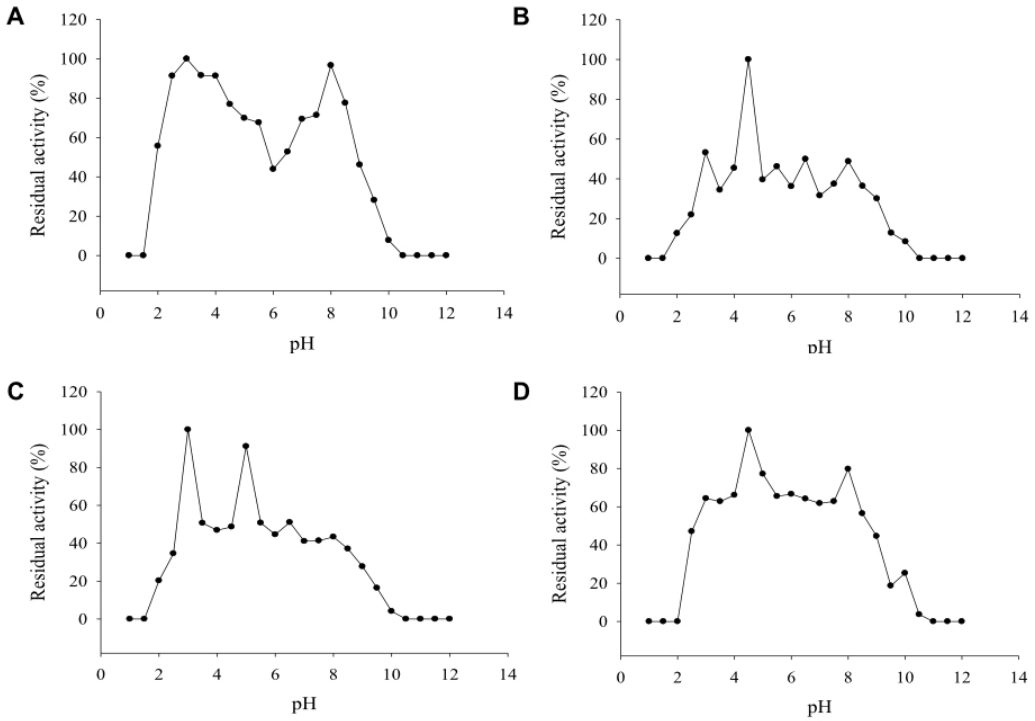

Figure 3. Optimal pH for the phytases derived from the four endophytic isolates. The optimal pH values were determined in phytase-inducing liquid media after 48 hours of culture. A - *P. aeruginosa* BAC3115, B - *S. maltophilia* BAC2135, C - *M. foliorum* BAC1157, D - *R. erythropolis* BAC2162.

To determine the specific phytase activity, different phosphate sources were used to measured phytase activity from the four endophytic bacteria isolates. Activity toward β-glycerophosphate and sodium pyrophosphate was detected in the culture supernatants of the four bacterial isolates when phosphate compounds and phytic acid were both used as substrates in the reaction. *S. maltophilia* BAC2135 was the only bacterial isolate that showed activity with ATP, while *M. foliorum* BAC1157 and *R. erythropolis* BAC2162 showed activity with ADP (Table 2). Bacterial phytases usually exhibit specificity for phytic acid and low activities toward other phosphorylated compounds, such as ADP, β-glycerophosphate and pyrophosphate (Shimizu, 1982; Casey and Walsh, 2003; Kim et al., 2003). As shown in Table 2, the enzymes produced by the endophytic isolates can be characterized as phytases and phosphatases with a narrow spectrum of action on other phosphorylated compounds.

Table 2. Phytase specificity for phosphorylated substrates.

Substrate	Relative activity (%)			
	BAC3115	BAC2135	BAC1157	BAC2162
Phytate	100 a	100 a	100 a	100 a
β-Glycerophosphate	35 c	13c	20 cd	59 b
ADP	0 d	0 c	48 b	53 c
ATP	0 d	15 c	0 d	0 e
Sodium pyrophosphate	73 b	60 b	32 bc	11 d

BAC3115 - *P. aeruginosa*, BAC2135 - *S. maltophilia*, BAC1157 - *M. foliorum*, BAC2162 - *R. erythropolis*. The values followed by the same letter in the same column do not differ in Tukey's test at 5% probability.

Results and discussion

The effects of 1, 5, 10 and 20 mM Ca^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{2+} and Zn^{2+} on the phytase activity of the four bacterial isolates can be found in Table 3. The phytase activity of the *P. aeruginosa* BAC3115 supernatant increased with the Ca^{2+} concentration, and 1 and 5 mM Cu^{2+}. In contrast, 10 and 20 mM Mn^{2+} and Zn^{2+} decreased the enzymatic activity of this isolate. Ten millimolar Ca^{2+} increased the phytase activity of the *S. maltophilia* BAC2135 isolate, although high Mn^{2+} and Zn^{2+} concentrations decreased the activity of this supernatant. The enzyme produced by *M. foliorum* BAC1157 showed the highest activity in high Ca^{2+} concentrations, 1 to 20 mM Fe^{2+}, high Mg^{2+} and low Mn^{2+} and Zn^{2+} concentrations. The phytase produced by *R. erythropolis* BAC2162 was the most active in high Fe^{2+} concentrations and low Mn^{2+} concentrations.

Table 3. Effect of divalent cations on the activity of endophytic isolate-derived phytases.

	Cation concentration	Ca^{2+} ($CaCl_2$)	Cd^{+2} ($CdSO_4$)	Cu^{+2} ($CuSO_4$)	Fe^{+2} ($FeSO_4$)	K^{+} (KCl)	Mg^{+2} ($MgCl_2$)	Mn^{+2} ($MnCl_2$)	Zn^{+2} ($ZnSO_4$)
BAC3115	0 mM	0.171 (±0.009) b	0.171 (±0.009) ns	0.171 (± 0.009) b	0.171 (±0.009) ns	0.171 (±0.009) ab	0.171 (±0.009) ns	0.171 (±0.009) a	0.171 (±0.009) ab
	1 mM	0.169 (±0.007) b	0.194 (±0.009) ns	0.239 (±0.016) a	0.179 (±0.011) ns	0.148 (±0.017) b	0.178 (±0.009) ns	0.159 (±0.003) a	0.181 (±0.007) a
	5 mM	0.183 (±0.008) ab	0.179 (±0.006) ns	0.240 (±0.021) a	0.178 (±0.005) ns	0.176 (±0.017) ab	0.174 (±0.004) ns	0.166 (±0.003) a	0.169 (±0.004) ab
	10 mM	0.183 (±0.001) b	0.198 (±0.015) ns	0.178 (±0.009) b	0.170 (±0.001) ns	0.172 (±0.002) ab	0.179 (±0.004) ns	0.061 (±0.009) b	0.158 (±0.002) bc
	20 mM	0.194 (±0.009) a	0.185 (±0.006) ns	0.154 (±0.011) b	0.178 (±0.008) ns	0.190 (±0.002) a	0.174 (±0.020) ns	0.000 (±0.000) c	0.142 (± 0.004) c
BAC2135	0 mM	0.150 (±0.002) cd	0.150 (±0.002) a	0.150 (±0.002) ab	0.150 (±0.002) ab	0.171 (±0.009) ab	0.150 (±0.002) ns	0.150 (±0.002) a	0.150 (±0.002) ab
	1 mM	0.144 (±0.008) d	0.116 (±0.018) c	0.162 (±0.005) a	0.140 (±0.007) b	0.148 (±0.017) b	0.141 (±0.010) ns	0.122 (±0.004) c	0.160 (±0.007) a
	5 mM	0.169 (±0.001) c	0.136 (±0.003) abc	0.142 (±0.009) ab	0.160 (±0.005) ab	0.176 (±0.017) ab	0.131 (±0.002) ns	0.146 (±0.001) ab	0.144 (±0.004) b
	10 mM	0.204 (±0.006) a	0.121 (±0.003) bc	0.138 (±0.005) b	0.151 (±0.013) ab	0.172 (±0.002) ab	0.166 (±0.024) ns	0.140 (±0.005) b	0.088 (±0.005) c
	20 mM	0.190 (±0.002) b	0.145 (±0.013) ab	0.143 (±0.006) b	0.165 (±0.002) a	0.190 (±0.002) a	0.153 (±0.025) ns	0.084 (±0.004) d	0.000 (±0.000) d
BAC1157	0 mM	0.189 (±0.003) cd	0.189 (±0.003) b	0.189 (±0.003) a	0.189 (±0.003) b	0.189 (±0.003) ns	0.189 (±0.003) b	0.189 (±0.003) a	0.189 (±0.003) c
	1 mM	0.156 (±0.012) d	0.205 (±0.009) ab	0.192 (±0.004) a	0.203 (±0.006) a	0.170 (±0.008) ns	0.225 (±0.042) ab	0.187 (±0.008) a	0.213 (±0.010) ab
	5 mM	0.166 (±0.012) c	0.221 (±0.009) a	0.176 (±0.009) a	0.193 (±0.004) ab	0.194 (±0.021) ns	0.211 (±0.009) ab	0.185 (±0.011) ab	0.225 (±0.003) a
	10 mM	0.197 (±0.005) a	0.166 (±0.004) c	0.180 (±0.013) a	0.198 (±0.007) ab	0.183 (±0.009) ns	0.220 (±0.035) ab	0.166 (±0.007) c	0.204 (±0.001) b
	20 mM	0.191 (±0.005) b	0.148 (±0.007) c	0.195 (±0.004) a	0.204 (±0.007) a	0.164 (±0.023) ns	0.269 (±0.009) a	0.168 (±0.004) bc	0.206 (±0.000) b
BAC2162	0 mM	0.179 (±0.001) ab	0.179 (±0.001) ab	0.179 (±0.001) ab	0.179 (±0.001) c	0.179 (±0.001) ab	0.179 (±0.001) c	0.179 (±0.001) b	0.179 (±0.001) a
	1 mM	0.122 (±0.007) c	0.187 (±0.005) a	0.190 (±0.015) a	0.149 (±0.004) d	0.183 (±0.004) a	0.201 (±0.003) b	0.225 (±0.005) a	0.072 (±0.007) b
	5 mM	0.168 (±0.008) b	0.163 (±0.005) c	0.178 (±0.005) ab	0.195 (±0.006) c	0.169 (±0.005) bc	0.199 (±0.005) b	0.211 (±0.011) a	0.022 (±0.014) c
	10 mM	0.177 (±0.002) b	0170 (±0.002) bc	0.178 (±0.002) ab	0.263 (±0.010) a	0.157 (±0.002) d	0.207 (±0.005) b	0.147 (±0.004) c	0.013 (±0.003) cd
	20 mM	0.191 (±0.001) a	0.180 (±0.001) a	0.167 (±0.005) b	0.214 (±0.009) b	0.159 (±0.007) cd	0.220 (±0.005) a	0.155 (±0.005) c	0.000 (±0.000) d

The large number of phytase-producing bacterial isolates found in this work suggests that endophytic bacteria are promising candidates for the production and study of this enzyme. While assessing the potential production of bacterial phytase in Malaysia, Hussin et al. (2009) reported that endophytic bacteria exhibited the highest phytase activity. Endophytic microorganisms can be vertically transmitted to seeds. Considering that phytic acid is the main storage form of Pi in grains and legumes, it is plausible that a selection of endophytic microorganisms capable of degrading phytic acid and assisting in the process of seed germination might exist (López-López et al., 2010).

Extracellular enzymes are excellent candidates for large-scale production and marketing because they eliminate the expenses associated with the extraction process (Anis Shobirin et al., 2009). The most production of bacterial phytases are intracellular, with the exception of those phytases that are derived from *Bacillus subtilis*, *Lactobacillus amylovorus* and *Enterobacter* sp. 4 species (Vohra and Satyanarayana, 2003). However, in the current study, those bacterial isolates presenting the largest hydrolysis halos around the colonies in solid media containing calcium phytate suggests an extracellular phytase activity. Anis Shobirin et al. (2009) also described two bacterial isolates of the *Pantoea stewartii* species as extracellular phytase producing bacteria. Consequently, the *Microbacterium*, *Pseudomonas, Rhodococcus* and *Stenotrophomonas* genera should be included among those genera with extracellular phytase activity.

To assess the potential application of the phytases produced by *P. aeruginosa* BAC3115, *S. maltophilia* BAC2135, *M. foliorum* BAC1157 and *R. erythropolis* BAC2162 in the animal feed industry, the enzymes produced by these bacteria were characterized for optimal activity pH and temperature, substrate specificity and divalent cations effect. The phytase produced by the *M. foliorum* BAC1157 isolate was the most active at 70 °C. Furthermore, the phytase in question showed 90% activity at 40 °C, which is another desirable feature, as the body temperature of pigs is 39 °C (Casey and Walsh, 2003). The optimal temperature for most microbial phytases is approximately 50 °C (Simon and Igbasan, 2002); however, some microbial phytases have exhibited optimal temperatures above 60 °C (Berka et al., 1998; Matsui et al., 2000; Zamudio et al., 2001). *M. foliorum* BAC1157 isolate has a remarkable application and can be used as a supplement in swine feed because the pelleting process involves temperatures above 70 °C.

The endophytic isolates used in the here exhibited more than one optimal pH peak, suggesting the production of phytases with different characteristics. *B. subtilis* (natto) produces a phytase with an optimal activity between 6.0 and 6.5 (Shimizu, 1992), and the *C. braakii* phytase shows optimal activity at a pH of approximately 4.0 (Kim et al., 1998). The phytase produced by *M. foliorum* BAC1157 showed the highest enzymatic activity at a pH of 3.0 and merely 20% activity at pH 2.0. The pH in the swine stomach varies between 2.0 and 4.0 (Lindberg and Ogel, 2001) depending on the feed and other factors. This substantial loss of activity at pH 2.0 is a negative factor, but controlling animal feed can ensure that the stomach pH does not reach this value. However, the low pH in gastric phase promotes the environment where phytate is really soluble and most susceptible to hydrolyses. Although for some fungal phytases it seems to be a huge problem because of the range of optimal activity at high pH. On the other hand, isolating and characterizing the gene encoding the *M. foliorum* BAC1157 phytase may aid in the development of site-directed mutagenesis strategies designed to increase enzymatic activity at pH values below 4.0, similar to the

phytase applied in the animal feed industry, as the produced by *Aspergillus niger* NRRL3135 (Mullaney et al., 2002).

Because the several divalent cations found in the diets administered to monogastric animals may interfere with the activity of supplemental enzymes added to these foods, it is necessary to predict the effect of such cations on phytase activity. The addition of cations to the enzymatic reaction changed the phytase activity of the four bacterial isolates. The *M. foliorum* BAC1157 bacterial isolate was only inhibited at high concentrations of Cd^{2+} and Mn^{2+}, in addition to showing phytase activity at 70 °C. Furthermore, increased phytase activity in the presence of Zn^{2+} is an uncommon feature, especially considering that this metal inhibits several of the phytases reported in the literature (Yoon et al., 1996; Greiner et al., 1997; Greiner et al., 2009). Studies have reported that the presence of Ca^{2+} did not affect the activity of *A. niger* 11T53A9 (Greiner et al., 2009) and *C. braaki* phytases (Kim et al., 1998). However, Ca^{2+} inhibited the activity of a phytase produced by *A. niger* ATCC9142 (Casey and Walsh, 2003), and decreased the activity of a phytase produced by *B. subtilis* DS11 (Kim et al., 1998). Similarly, Fe^{2+} and Fe^{+3} did not affect the activity of the phytase produced by *A. niger* ATC9142 (Casey and Walsh, 2003), despite inhibiting some other phytases (Kim et al., 1998; Greiner et al., 2009). Increased activity in the presence of cations such as Cu^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} was another unusual feature noted in the phytase isolated by Casey and Walsh (Casey and Walsh, 2003).

Conclusion

The results of the current study suggest that *P. aeruginosa* BAC3115, *R. erythropolis* BAC2162, *S. maltophilia* BAC2135 and *M. foliorum* BAC1157 are promising candidates for the study and application of endophytic isolate-derived phytases in the feed industry. To our knowledge, this is the first report on phytase production by the bacteria *Microbacterium foliorum* BAC1157, in which this phytase could has a potential use in monogastric diets.

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Conflict of interest

The authors declare that they have no conflict of interest.

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