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PURIFICATION AND PARTIAL CHARACTERIZATION OF MANGANESE PEROXIDASE FROM *Bacillus pumilus* AND *Paenibacillus* sp.

[1]Patrícia Lopes de Oliveira*; [1]Marta Cristina Teixeira Duarte; [1]Alexandre Nunes Ponezi, [2]Lúcia Regina Durrant

[1]Divisão de Microbiologia, Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas, Campinas, SP, Brasil. [2]Laboratório de Sistemática e Fisiologia Microbiana, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, SP, Brasil.

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ABSTRACT

The production of manganese peroxidase (MnP) from *Bacillus pumilus* and *Paenibacillus* sp. was studied under absence and presence of the inducers indulin AT, guayacol, veratryl alcohol, lignosulfonic acid and lignosulfonic acid desulfonated. Indulin AT increased the activity of *B. pumilus* MnP up to 31.66 U/L after 8 h, but no improve was observed for *Paenibacillus* sp., which reached maximum activity (12.22 U/L) after 20 h. Both MnPs produced by these microorganisms were purified in phenyl sepharose resin and the proteins from crude extracts were eluted in two fractions. However, only the first fraction of each extract exhibited MnP activities. Tests in different pH and temperature values, from pH 5.0 to pH 10.0 and 30 ºC to 60 ºC, respectively, were carried out with the purified MnP. The maximum activity reached for *B. pumilus* and *Paenibacillus* sp. MnPs were 4.3 U/L at pH 8.0 and 25 ^{o}C and 11.74 U/L at pH 9.0 and 35 ^{o}C, respectively. The molar masses determined by SDS-PAGE gel eletrophoresis were 25 kDa and 40 kDa, respectively, for the purified enzyme from *B. pumilus* and *Paenibacillus* sp.

Key words: *Bacillus pumilus*; *Paenibacillus* sp.; Manganese peroxidase; Purification; Characterization.

INTRODUCTION

Plant cell walls have lignin in their structure as the most abundant component. Lignin is an aromatic and heterogeneous constituent that ensures strength and resistance towards microbial attack. White-rot basidiomycetes are the most common organisms known to efficiently degrade and mineralize lignin into CO_2 and H_2O, due to extracellular enzymes involved in lignin degradation, particularly the ligninase complex, formed by laccase, lignin peroxidase and manganese peroxidase (10, 11). Rarely these three enzymes are present in the same organism, and different combinations of them can operate. The ligninase complex is frequently produced during secondary metabolism but different species have particular responces to nutrients (19, 25).

Due to the important degradative potential of manganese peroxidase (MnP), there is a general interest in producing the enzyme biotechnologically. Manganese peroxidase, a glycosylated heme-containing enzyme, have been used besides biodegradation of lignin (13), in the biodegradation

***Corresponding Author. Mailing address:** Divisão de Microbiologia - Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, CPQBA-UNICAMP, Caixa Postal 6171, CEP 13083-080, Campinas, SP, Brasil.; Email: patricia_lopes13@yahoo.com.br

of polycyclic aromatic hydrocarbons (PAH) (24, 27), humic acids (28), synthetic dyes (12), and polychlorinated biphenyls (PCB) (2). MnP oxidizes Mn^{2+} to Mn^{3+} in an H_2O_2-dependent reaction and Mn^{3+} is stabilized by chelating dicarboxylic acids (8).

The optimum pH of almost all ligninolytic enzymes, including MnP, reported to date lies in the acidic range. However some industry activities, such as pulping and bleaching are mainly performed under highly alkaline conditions and the waste generated is also alkaline. Ligninolytic enzymes having acidic optimum pH values cannot be used under alkaline conditions (16).

Few reports on bacterial MnP are found in the literature. Two bacteria, *B. pumilus*, isolated from wood decomposition material by Duarte *et al*. (6) and *Paenibacillus* sp. isolated from paper mill effluent (22) were able to produce MnP in alkaline conditions. These enzymes were able to remove the color from paper mill effluent (23). In this study, we report on the purification and partial characterization of MnP from *B. pumilus* and *Paenibacillus* sp.

MATERIALS AND METHODS

Microorganisms

Bacillus pumilus CBMAI 0008 was isolated from wood decomposition material by Duarte *et al*. (6) and was maintained in a culture medium containing birchwood xylan (20).

Paenibacillus sp. CBMAI 868 was isolated from paper industry effluent, in a media containing 1% birchwood xylan (Sigma); 0.1% $(NH_4)_2SO_4$; 50% paper mill effluent; and 2% agar-agar. After sterilization, nistatin (0,044 mg/mL) was added as an antifungal control. An aliquot of 0.3 mL from paper mill effluent was used to sow the surface medium in Petri plates. After incubation (37 ^oC, 48 h), the ability of colonies to grow and produce clear haloes of decolorization on the medium surface was verified. The colony that provided the greater clear haloe was purified and identified at Microbial Resources Divison – CPQBA/UNICAMP.

The subsequent assays were carried out at 45 ^oC for both bacteria, once this temperature is close to that used in several industrial processes.

Inocula preparation

The isolates were individually transferred to 125 mL Erlenmeyer flasks, containing 12.5 mL of the liquid media (20), and incubated at 45 ^oC in a shaker (250 rpm) for 20 h. The culture was centrifuged in aseptic conditions and the cells were ressuspended in 40 mL of the media. Further steps of MnP production were carried out using inoculum at 8 % (v/v).

Effect of inducers on the MnP production

For cinetic studies, inocula were prepared as described above and the fermentation was carried out in 250 mL Erlenmeyer flasks, containing 50 mL of the liquid media, and incubated at 45 ^oC in a shaker (250 rpm) during 32 h. At regular periods, samples were collected for MnP activity measurement. MnP production was also studied in the presence of veratryl alcohol, industrial lignin (indulin AT), guayacol, lignosulfonic acid and lignosulfonic acid desulfonated at 0.1% (w/v) at the same conditions. The fermented media was centrifuged for 15 min at 12000 x g for the activity assays.

Enzyme activity assay

Manganese peroxidase activity was assayed spectrophotometrically according to Kuwahara (17). The reaction mixture contained 0.1 mL of 0.25 M sodium lactate, 0.05 mL of 2 mM $MnSO_4$, 0.2 mL of 0.5% serum albumin bovine, 0.1 mL of 0.1% phenol red, 0.5 mL enzyme and 0.05 mL of 2 mM H_2O_2 in 0.2 M sodim phosphate buffer (pH 8.0). The mixture was left at room temperature for 5 min and the reaction was ended with 0.04 mL NaOH 2 N. The absorbance was read at 610 nm and the activity expressed in U/L. One activity unit was defined as amount of enzyme necessary to oxidize 1 μmol of substrate per minute.

Proteins measurement

Protein concentration was measured by the Bradford method (3). Bovin serum albumin was used as a standard. The results were used to calculate specific activity.

Culture conditions for crude extracts production aim purification and characterization

The culture conditions for crude extracts production aim purification and characterization were determined after inducers studies. The bacteria were cultured as described above for inoculum production and subsequently inoculated in the medium containing xylan (20) enriched with indulin AT (*B. pumilus*) or without inducer (*Paenibacillus* sp.), and incubated at 45 ^{o}C in a shaker (250 rpm) for 8 h or 20 h, respectively. The cultures were centrifuged during 15 min at 12000 x g prior the purification assays.

MnP purification and partial characterization

All purification procedures were done at room temperature. The enzyme activity and protein concentrations were determined in all steps. The supernatant obtained from the crude broth was filtered (Whatman 0.45 μm) and futher concentrated in a Pellicon™ ultrafiltration system using 10 kDa Biomax 10™ membrane. The concentrated solution was loaded to a Pharmacia KX-26 40/26 column packed with Phenyl Sepharose hydrophobic interaction chromatography (HIC) resin coupled to a Pharmacia FPLC, previously equilibrated with 1.7 M $(NH_4)_2SO_4$ (9). The flow rate was 3 mL/min and proteins were eluted in a linear $(NH_4)_2SO_4$-gradient from 1.7 to 0 M in sodium phosphate buffer, pH 7.0, 50 mM. The peaks containing MnP activity were pooled, concentrated and dialyzed against the same buffer. The HIC-separated MnP was further loaded to a Pharmacia KX-16 16/2 column packed with Q-Sepharose anion exchange chromatography resin coupled to a Pharmacia FPLC, previously equilibrated with sodium phosphate 50 mM. The flow rate was 2 mL/min and proteins were eluted with sodium phosphate 50 mM in a linear NaCl-gradient from 0 to 1 M, pH 7.0. The peaks were all collected in a Red Frak™ (Pharmacia) system, monitored at 280 nm.

Electrophoresis – SDS PAGE

Purity and apparent molecular weights of the peaks were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (18) using a Mini-Protean II system (Biorad). Molecular weight markers (Promega) were included in 10% gels, followed by Comassie Blue R-250 staining.

Effect of pH and temperature on MnP activity

The effect of pH on purified MnP activity from *B. pumilus* and *Paenibacillus* sp. was studied in the following buffers (200 mM): citrate phosphate, pH 5.0 and pH 6.0; sodium phosphate, pH 7.0 and pH 8.0; and glycine-NaOH, pH 9.0 and pH 10.0. The effect of temperature was determined in range from 25 ^{o}C to 60 ^{o}C with 5 ^{o}C intervals and the incubation was according to Kuwahara *et al*. method (17).

RESULTS AND DISCUSSION

Effect of inducers on the production of MnP

The production of MnP was determined in the crude extract of *B. pumilus* and *Paenibacillus* sp., in the absence and presence of inducers. The maximum activity reached for the *B. pumilus* MnP without inducers was 6.41 U/L after 16 h (Figure 1-A). When the inducers were added in the culture medium, the activity increased to 31.66 U/L in the presence of indulin AT after 8 h (Figure 1-B), followed by lignosulfonic acid (15.6 U/L) and lignosulfonic acid desulfonated (8.6 U/L) after 6 h (Figures 1-D and 1-E, respectively). The maximum MnP production by *Paenibacillus* sp. was 13.76 U/L in the presence of veratryl alcohol after 28 h (Figure 1-C). The addition of lignosulfonic acid desulfonated resulted in a maximum activity of 12.78 U/L at the same time (Figure 1-D), while maximum activity in the presence of guayacol was 12.20 U/L after 20 h (Figure

1-F). These results did not show improve in the *Paenibacillus* sp. MnP activity since the activity without inducers was 12.33 U/L after 20 h (Figure 1-A). No data was found in the literature about the use of inducers in the MnP production from bacteria. The use of Polyfon H as inducer in the MnP production by the fungus *Irpex flavus* allowed reaching 0.08 U/mL. Inducers absence in the media was the best conditions for MnP production by *Dichomitus squalens* (0.29 U/mL) and *Polyporus sanguineus* (0.43 U/mL) (7). Further study revelead that indulin AT, Polyfon H, Reax 80, Orzan S, veratryl alcohol and guayacol did not increase MnP activity by *Phlebia floridensis*, a white-rot fungus (1).

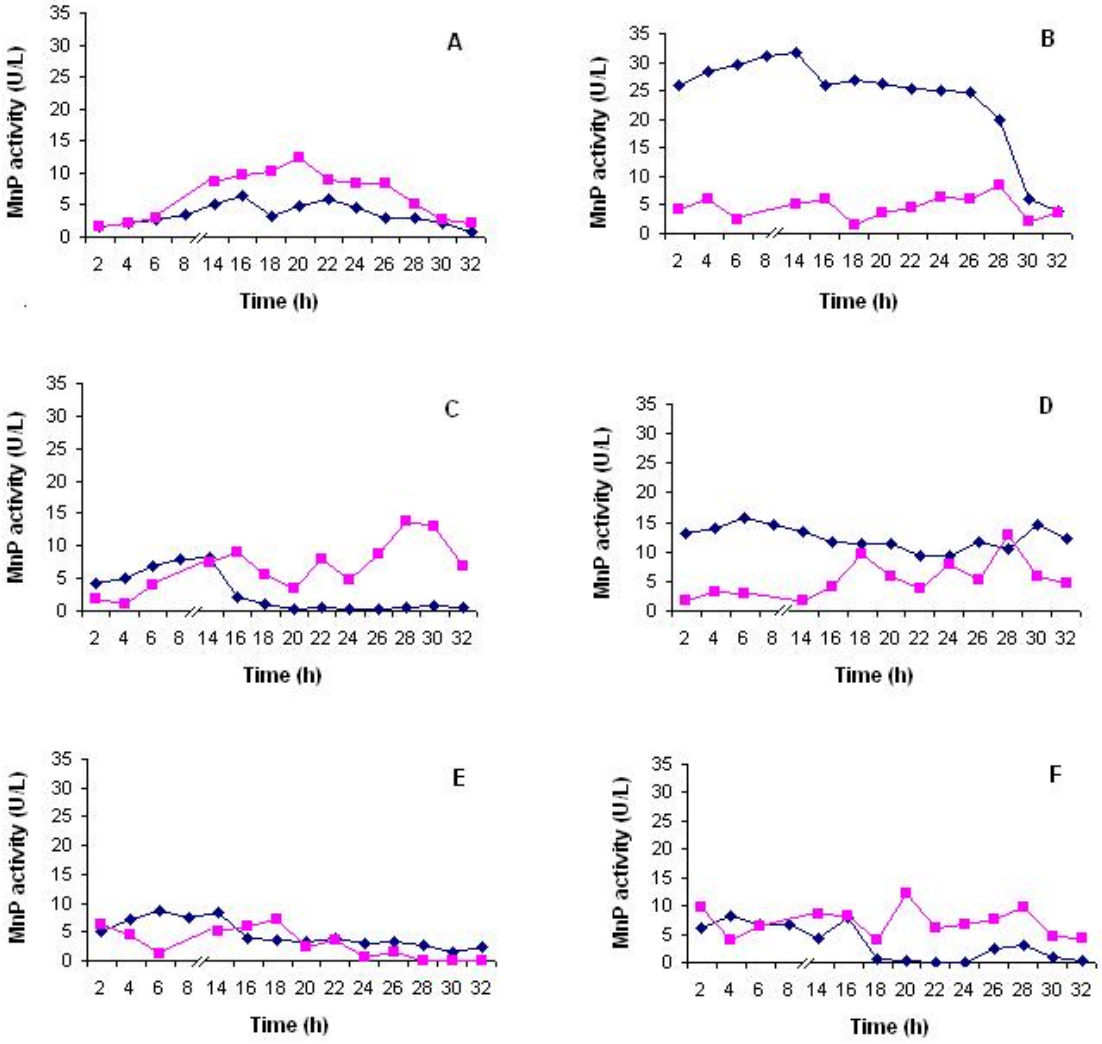

Figure 1. MnP production by *B. pumilus* CBMAI 0008 (◆) and *Paenibacillus* sp. CBMAI 868 (■). A – no inducers; B – indulin AT; C – veratryl alcohol; D – dissulf lignin acid; E – lignin acid and F – guayacol.

Enyme purification

The crude extract produced by *B. pumilus* and *Paenibacillus* sp. was first taken through the hydrophobic interaction resin (phenyl sepharose) obtaining two peaks as shown in Figures 2-A and 2-B. Subsequently, the eluted peaks were submitted to ultrafiltration and further purification in Q-Sepharose anion exchange chromatography resin, confirming the presence of only one proteic fraction. In all purification steps, MnP activity and total proteins were determined. A summary of the purification steps is shown in Table 1.

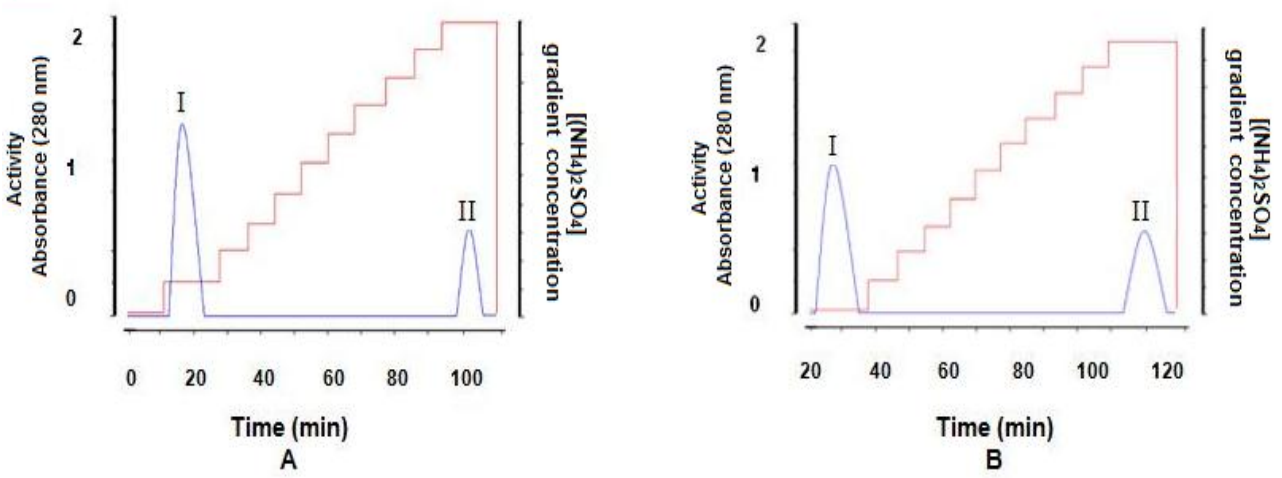

Figure 2. FPLC chromatogram in a Fenil Sepharose hydrophobic interaction resin. A – *B. pumilus* CBMAI 0008 MnP elution characterized in peak I. B – *Paenibacillus* sp. CBMAI 868 MnP elution characterized in peak I. Activity (———) and gradient concentration (———).

Table 1. Purification steps of MnP from *B. pumilus* CBMAI 0008 and *Paenibacillus* sp. CBMAI 868.

Purification step	Total volume (mL)	MnP activity (U/L)	Total protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Fold
		B. pumilus				
Fenil Sepharose	140	0.43	0.080	5.37	100.00	1.00
Ultraf./Dial.	14	4.20	0.138	30.39	976.74	5.66
Q-Sepharose	80	0.38	0.013	29.23	88.37	5.44
Ultraf./Dial.	8	3.45	0.110	31.36	802.33	5.84
		Paenibacillus sp.				
Fenil Sepharose	110	0.35	0.053	6.60	100.00	1.00
Ultraf./Dial.	11	3.20	0.317	10.09	914.29	1.53
Q-Sepharose	70	0.28	0.044	6.36	80.00	0.96
Ultraf./Dial.	7	2.40	0.284	8.45	685.71	1.28

Electrophoresis – SDS PAGE

The purified MnP from *B. pumilus* and *Paenibacillus* sp. appeared as single bands on 10% SDS-PAGE and presented molecular weights of 25 kDa and 40 kDa, respectively (Figure 3). MnP-PGY and MnP-GY produced by fungus *Pleorotus ostreatus* (15), and a purified MnP from *Trametes versicolor* (4) revelead molecular weights of 42 kDa close to the molecular weight found for *Paenibacillus* sp. in the present study. Also Hoshino *et al*. (14) verified a similar molecular weight of 40 kDa for MnP produced by fungus *Lenzites betulinus*. According to this study, the purified MnP from *B. pumilus* showed inferior molecular weights comparing to those produced by fungus reported in the literature.

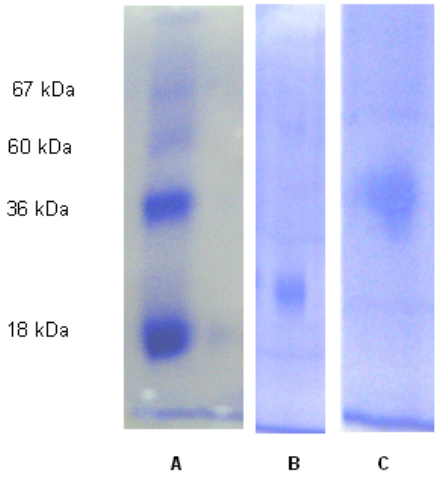

Figure 3. SDS electrophoresis: A – molecular weight standards; B – purified MnP from *B. pumilus* CBMAI 0008; C – purified MnP from *Paenibacillus* sp. CBMAI 868. Coomassie Brilliant Blue staining.

Effect of pH and temperature on MnP activity

The results of enzymatic activity in different pH and temperature values for purified MnP from *B. pumilus* and *Paenibacillus* sp. are shown in Figures 4-A and 4-B. The activity of *B. pumilus* MnP increased according to pH from 5.0 to 8.0, where occurred the maximum activity (4.3 U/L), and decreased at superior pH values (Figure 4-A). Optimum activity for *Paenibacillus* sp. was observed at pH 9.0 (5.65 U/L). MnP produced by this bacteria showed greater stability in different pH values than MnP produced by *B. pumilus*, since the lowest activity was at pH 6.0 (3.56 U/L) (Figure 4-A). Since the isolation conditions of these microorganisms were carried out in alkaline media, these results confirmed the maximum activities of purified enzymes in these conditions. Tests aiming the determination of optimum temperature values were done at best pH conditions for both microorganisms. Higher MnP activity occurred at 25 ºC for *B. pumilus* (4.3 U/L) and 35 ºC for *Paenibacillus* sp.(11.74 U/L), (Figure 4-B). MnP activity was not detected at 55 ºC and above (Figure 4-B).

The MnP optimum temperature is variable according to microorganism, as verified in previous studies. Purified MnP from *Aspergillus terreus* LD-1 showed maximum activity at 37 ºC (16), while the optimum temperature for the enzymes produced by *Dichomitus squalens*, *Irpex flavus* and *Polyporus sanguineus* was 30 ºC. In these cases, the enzyme was not detectable at 35 ºC, except for MnP from *Polyporus sanguineus* (7). In the present study, the optimum temperature for purified MnP from *B. pumilus* and *Paenibacillus* sp. is the same verified, respectively, for *Phebia floridensis* (1) and *Schizophyllum* sp. F17 (5).

We concluded that MnP activity obtained from the crude extract from *B. pumilus* (31.66 U/L) was almost three times above that found for *Paenibacillus* sp. (12.33 U/L).

Recent researches indicate multiples biotechnological applications for fungi MnP. A few mentions on bacterial MnP are found in the literature. A summary on MnP fungi production comparing with the results obtained in the present study is shown in Table 2. The data show that some fungi spend a longer time (around 8 days) for enzymes production than observed for the bacteria studied in the present work. Therefore, this suggests that may be possible to reach the same activities if we cultured the bacteria during the same period.

In this study, we chose the alkaline effluent from paper industry to cultivate and isolate microorganisms which could grow and secretes useful alkaline lignin-degrading enzymes. Thus, we have purified two alkaline MnPs, respectively from *B. pumilus* and *Paenibacillus* sp., and we believe that this is the first report on the bacterial ligninolytic enzymes. Subsequent tests carried out aimed the color removal from paper mill effluent showed a decrease in the compounds responsible for the colour and confirmed that the compounds present in the paper effluent were depolymerized during the treatment (23), indicating an important application area for these enzymes.

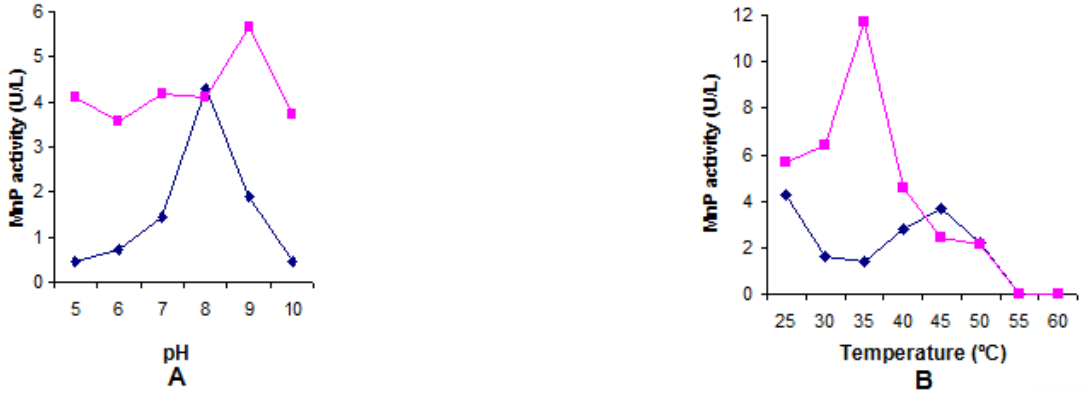

Figure 4. MnP activity from *B. pumilus* CBMAI 0008 (—◆—) and *Paenibacillus* sp. CBMAI 868 (—■—). A – Different pH values, buffers (0.2M): citrate phosphate, pH 5.0 and pH 6.0; sodium phosphate, pH 7.0 and pH 8.0; glycine-NaOH, pH 9.0 and pH 10.0. B - Different temperature values: *B. pumilus*, at pH 8.0 - sodium phosphate buffer 0.2 M and *Paenibacillus* sp., at pH 9.0
- glycine NaOH buffer 0.2 M.

Table 2. MnP activity found in the literature from different microorganisms.

Microorganisms	Culture and enzyme production conditions	Activity (U/L)	References
Dichomitus squalens	Malt extract broth, pH 5.5 at 25 °C, after 8 days	290	7
Irpex flavus	Mineral salts broth (MSB) with rice straw, pH 5.5 at 25 °C, after 8 days	340	
Polyporus sanguineus	Malt extract broth, pH 5.5 at 25 °C, after 8 days	430	
Pleurotus ostreatus	Yeast extract medium, peptone/glucose, pH 7.5 at 28 °C, after 8 days	740	15
Phlebia floridensis	N-limited MSB broth, pH 4.5 at 25 °C, after 4 days	60	1
Ganoderma sp.	Wheat bran, yeast extract, glucose and ammonium chloride, at 30 °C, after 7 days	7.8	26
Trametes versicolor	Glucose, pH 6.0 at 25 °C, after 8 days	44	21
Bacillus pumilus	Xylan medium with indulin AT, pH 9.0 at 45 °C, after 8h	31.66	PRESENT STUDY
Bacillus pumilus	Purified MnP, pH 8.0 at 25 °C	4.3	
Paenibacillus sp.	Xylan medium, pH 9.0 at 45 °C, after 20 h	12.33	
Paenibacillus sp.	Purified MnP, pH 9.0 at 35 °C	11.74	

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