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Full Length Research Paper

Pleurotus albidus: A new source of milk-clotting proteases

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Researches have been done to discover new sources of microbial proteases with milk-clotting activity to replace the traditional rennet from calves. The aim of this study was to select a species of edible mushroom as milk-clotting enzymes producer using the submerged fermentation technology. From the six species evaluated, *Pleurotus albidus* was the one that expressed the highest value of coagulant ratio (21.60). The milk-clotting enzymes showed maximum activity at 60°C and pH 6.0. Iodoacetic acid presented the highest inhibition in proteolytic activity suggesting the presence of cysteine proteases in the crude extract from *P. albidus*. The enzymes did not present toxic action against human fibroblasts (MRC5) in the analyzed conditions and, for that reason, can be suitable to applications in food industry. This is the first report of milk-clotting enzymes production by the edible mushroom *P. albidus*

Key words: *Pleurotus albidus*, milk-clotting, submerged fermentation.

INTRODUCTION

Proteolytic enzymes represent 65% of worldwide enzymes industry market and, among them, chymosin has great application in dairy industry acting in the casein peptides linkages during the cheese making (Yin et al., 2014). The raising of world cheese production, the shortage and the high price of tradition rennet associated with the rejection of proteases genetically modified has encouraged the search of new milk-clotting sources, especially the ones from microbial source (Ahmed et al., 2010; El-Baky et al., 2011; Leite Júnior et al., 2014; Wang et al., 2015).

The microbial protease presents advantages when compared with the enzymes from plants and animal due their biochemical diversity, possibility of genetic

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manipulation and low production costs (Erjavec et al., 2012; Savitha et al., 2011).

Fungi belong to the microbial diversity and have many advantages as considered by Generally Regarded as Safe (GRAS) and their extracellular enzymes are easily recovered in bioprocess. Among fungi, the edible mushrooms are macro fungi that already produce milk-clotting enzymes usually obtained from submerged fermentation (SmF) which is the most useful method for enzymes production. The advantages of SmF are the controlling of parameters as pH, temperature, oxygen and humidity besides the possibility of large-scale production (Sandri et al., 2015; Uzuner and Cekmecelioglu, 2015; Sandhya et al., 2005).

Due to the nutritional and medicinal properties of edible mushrooms, the uses of these fungi in food and pharmaceutical industries have been raising. Among the 7000 mushrooms species that present variable edible levels only 2000 of them are considered as safe. However, a reduced number of mushrooms species are considered dangerous because they produce toxic substances which are malefic to human health and can be lethal to the individual (Jo et al., 2014).

The aim of this study was to investigate the production of milk-clotting enzymes from mushrooms and select one species to determine their enzyme profile and application in cheese production.

MATERIALS AND METHODS

Mushrooms

Pleurotus albidus DPUA1692, *Auricularia mixotricha* DPUA 1695, *Ganoderma lucidum* DPUA 1694, *Lentinus citrinus* DPUA 1535, *Lentinus crinitus* DPUA 1693, and *Pleurotus ostreatoroseus* DPUA 1720 (Culture Collection DPUA/UFAM) were evaluated as milk-clotting enzymes producers. Each culture were cultivated in Potato Dextrose Agar (PDA medium and 0.5% (w/v) yeast extract) in Petri dishes. The cultures were maintained at 25°C for 12 days.

Fermentation media and culture conditions

The production of milk-clotting enzymes was made by submerged fermentation using liquid medium composed of (g/L): glucose (20), yeast extract (5), meat peptone (5), and gelatin (5) at pH 5.6. The fermentation was performed in 125 ml Erlenmeyer flasks containing 50 ml of sterilized medium (121°C for 15 min). After cooling, each flask was inoculated using 10 micelial discs of 5 cm diameter and incubated at 30°C, on rotatory shaker (Nova Técnica, model 712, Piracicaba, São Paulo, Brazil) at 150 rpm during 72 h. The biomass was separated from the crude extract by vacuum filtration on Whatman no. 1 paper filter.

Proteolytic activity assay

The proteolytic activity was determined according to Leighton et al. (1973). Protease activity was determined in the crude extracts (150 µl) using 1.0% (w/v) azocasein (250 µl) in 0.2 M Tris-HCl buffer, pH 7.2. One unit of proteolytic enzyme was defined as the amount of enzyme that produces a 0.1 increase of absorbance in 1 h at 440 nm (U/ml). All samples were prepared in triplicate.

Milk-clotting protease assay

Milk-clotting activity was determined according to Arima et al. (1970) using 10% (w/v) bovine skimmed milk poder (Itambé®) in 0.05 M $CaCl_2$ as substrate. Briefly, 5 ml of milk solution were distributed in test tubes and pre-incubated in water bath (Gant, model 179, Cambridge, England) at 40°C for 15 min. The enzyme extract (0.5 ml) was added to the milk and counting time started. Clot formation was observed while manually rotating the test tube. The time at which the first particles were formed was measured. All samples were prepared in triplicate. One unit of milk-clotting activity (U) was defined as the amount of enzyme required to coagulate 1 ml of substrate in 40 min at 40°C. Milk-clotting activity was calculated according to Shata (2005): $U = 2400/T \times S/E$, where T (s) is the necessary time to clot formation, S is the volume of milk (ml) and E is the volume of crude extract used (ml). The coagulant ratio (R) was calculated according to the ratio of milk-clotting activity and proteolytic activity values.

To select a strain and characterize its enzymes, the samples were grouped into three classes according to the formation of compact milk clot and milk whey separation in the test tube: strong milk coagulation (distinct clot and abundant whey); weak milk coagulation (clot formation without clear separation of the whey); and milk without coagulation (clot and whey absent) (Alecrim et al., 2015).

Effect of pH and temperature on activity and stability of milk-clotting enzymes

To assay optimum pH, proteolytic activity was determined at 40°C at different pH values using the following 0.1 M buffer solutions: sodium acetate (5.0 and 6.0), Tris-HCl (7.0 and 8.0) and Glicine-NaOH (9.0 and 10.0). Optimum temperature was determined by incubating the enzyme extract at different temperatures ranging from 30 to 80°C and assaying the activity at the pH determined as optimum.

For the pH stability, the crude extract was dispersed (1:1) in the following 0.1 M buffer solutions: sodium acetate (5.0 and 6.0), Tris-HCl (7.0 and 8.0) and Glicine-NaOH (9.0 and 10.0) and maintained at 25°C for 24 h. In thermal stability, the extracts were incubated at different temperatures ranging from 30 to 80°C for 1 h. The solution of 10% (w/v) skimmed milk powder in 0.05 M $CaCl_2$ was used as substrate. All samples were prepared in triplicate. Residual enzyme activities were determined according to the optimal conditions of pH and temperature.

Effect of inhibitors and metallic ions on milk-clotting activity

The effect of inhibitors and metallic ions on milk-clotting activity was investigated by using 1 mM of pepstatin A and 10 mM of phenylmethylsulfonyl fluoride (PMSF), ethylene-diaminetetraacetic acid (EDTA), iodoacetic acid, $CaCl_2$, $CuSO_4$, KCl, $FeSO_4$, $MgSO_4$, $MnSO_4$, NaCl and $ZnSO_4$ in the reaction. Samples were incubated at 40°C for 30 min and the milk-clotting activity was determined according to the methodology discribed previously. Residual enzyme activities were determined and compared to the control, which was incubated without the inhibitors and metallic ions and corresponds to 100% of activity. All samples were prepared in triplicate.

Toxicity test

All tests were carried out at the Biological Activity Laboratory, Pharmaceutical Science College, Federal University of Amazonas.

Strain and culture conditions

In this study, human fibroblasts (MRC5) maintained in culture bottles containing Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% (w/v) fetal bovine serum (FBS) and 1% (w/v) ampicillin-streptomycin were used. The cells were maintained in bacteriological incubator with 5% CO_2 at 37°C. This study model promoted evaluation of the cytotoxic effect of *P. albidus* crude extract, at different concentrations, against strains of MRC5.

***In vitro* cytotoxicity**

The cytotoxicity of *P. albidus* crude extracts was evaluated by Alamar blue test, according to Ahmed et al. (1994). Alamar blue (resazurin), a blue dye and no fluorescent was reduced to resofurin, pink colored and fluorescent, by the viable cells. The cells were transferred to a micro plate (96 wells) in concentration of 0.5×10^4 cells/well. These plates were maintained in incubator with 5% CO_2 for 24 h. The cells were then treated in single concentration of *Pleurotus* crude extract (200 µg/ml diluted in DMSO) for 72 h. The positive control was doxorubicin (1720 µM) and the negative control was 0.1% (w/v) DMSO. Three hours before each treatment was added 10 µl of 0.2% (v/v) Alamar blue and followed by readings at 465 and 540 nm using a micro plate reader. All the assays were performed in triplicate. The data were analyzed in GraphPad prism 6.0 program and images were captured by Zen 2 program from Carl Zeiis Microscopy Gmbh Company®.

Statistical analysis

In all experiments, the data were subjected to descriptive statistical analysis of variance and the averages were compared by Tukey's test ($p<0.05$) using Minitab software, version 16.0. (Minitab, 2010).

RESULTS AND DISCUSSION

The quality of the enzymes used in cheese making has influenced the milk compounds biotransformation and in the process of clot formation, which determines the sensorial properties of the final product. Proteases with similar activities to the commercial enzymes were already identified in Basidiomycetes (Shamtsyan et al., 2014).

Another important parameter is the coagulant ratio. The relation of milk-clotting activity and proteolytic activity is associated with the enzymatic specific in the clot formation and influences in the selection of a new source of milk-clotting enzymes (Alecrim et al., 2015).

From the six edible mushrooms selected in this study, *G. lucidum* and *P. albidus* were the species that produced milk-clotting enzymes. The values of milk-clotting activity and coagulant ratio were significative to *P. albidus* (73.39 U/ml and 21.60, respectively). Silva et al. (2014) reported milk-clotting activity (60.5 U/ml) and coagulant ratio (510) in *Thermomucor indicae-seudaticae* N31. In the study of Alecrim et al. (2015), *Aspergillus flavo furcatis* DPUA 1493 presented milk-clotting activity of 68.61 U/ml and coagulant ratio of 1.81. Milk-clotting enzymes from *Termitomyces clypeatus* presented values of 333.33 U/ml and 64.70 of milk-clotting activity and coagulant ratio, respectively, in the study of Majumder et al. (2015).

Figure 1. Milk-clotting activity from *P. albidus* crude extract.

The milk-clotting enzymes from *P. albidus* formed a clot classified as strong, forming abundant whey after the addition of the enzymatic extract in milk solution (Figure 1). Similar result of clot formation was reported in the study of Alecrim et al. (2015) with enzymes from *A. flavo furcatis*, an anomorphic fungus.

The studies of optimum conditions of pH, temperature and stability can be used as indicatives of enzymes industrial application (Castro and Sato, 2013). The effect of pH in the activity of *P. albidus* milk-clotting enzymes is as shown in Figure 2. The raising of pH promoted the loss of milk-clotting activity. The enzymes exhibited 100% of activity at pH 6.0 and at pH 10.0 the activity was only 19.37%.

Pediococcus acidilactici SH enzymes also showed high milk-clotting activity at pH 6.0, however, it was observed that at pH 7.0 and 8.0 the activity decreased in 54 and 21%, respectively (Imdakim et al., 2015). Vishwanatha et al. (2010) reported high milk-clotting activity value at pH 6.3 from the enzymes produced by *Aspergillus oryzae* MTCC 5341 followed by the reduction of the activity in high values of pH.

In conditions out of the optimum pH, there was loss of enzymatic activity due to conformation changes in the protein structure caused by charges repulsion. The distribution of charge in the protein surface and the conformations are modified and, for that reason, the enzymes cannot be associated with the substrate correctly. According to Vasconcelos et al. (2004) the pH can influence in the yield of milk-clotting. In the ranges of pH from 5.7 to 6.2, the milk-clotting enzymes from microbial source present higher proteolytic activity when compared with the ones from animals and genetic modified.

The temperature had great influence in the milk-clotting

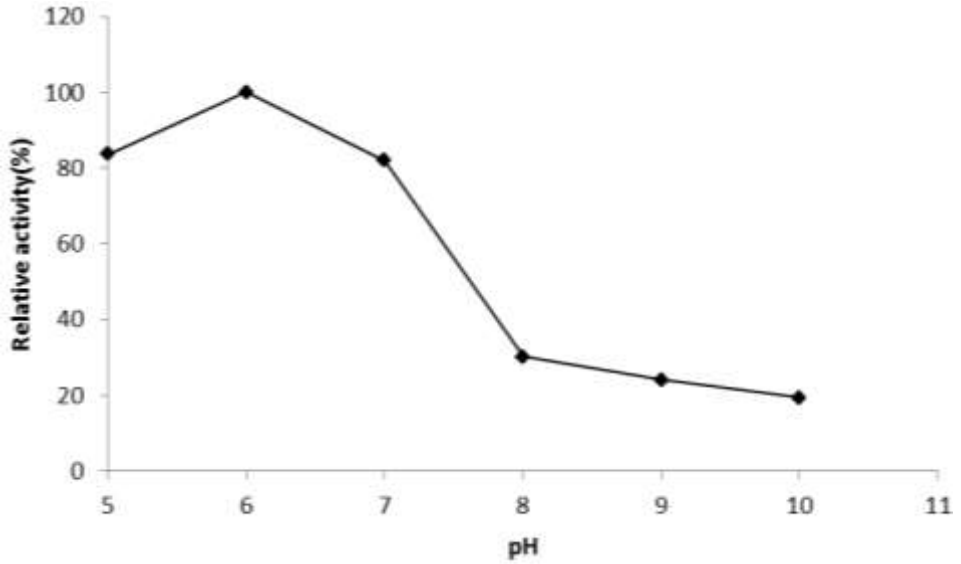

Figure 2. Effect of pH in milk-clotting activity from *P. albidus* crude extract.

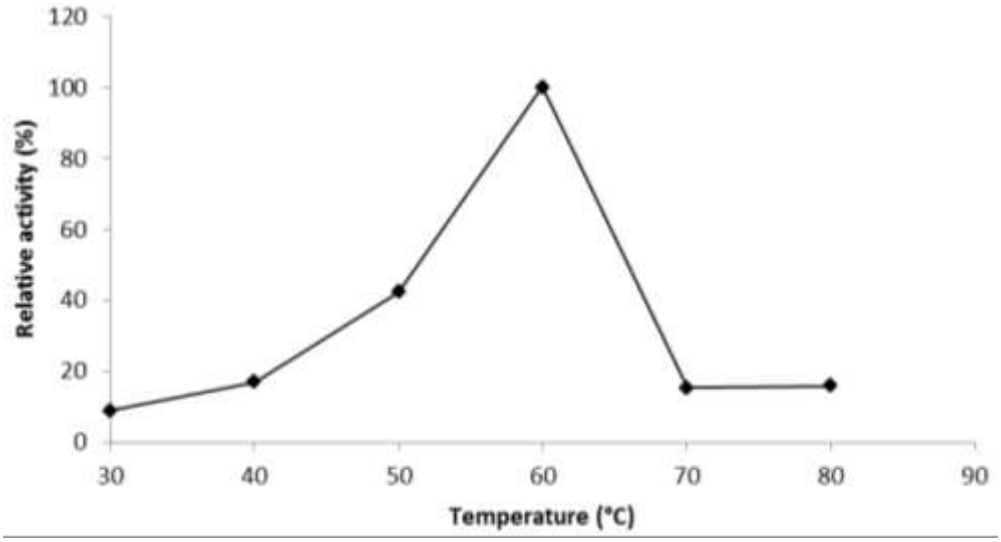

Figure 3. Effect of temperature in the milk-clotting activiy from *P. albidus* crude extract.

enzymes activity (Figure 3). The optimum temperature was determined at 60°C. Milk-clotting enzymes from other fungi as *Rhizopus oryzae* (Kumar et al., 2005), *Penicillium oxalicum* (Hashem, 1999) and *Rhizopus microsporus* var*. rhizopodiformis* (Sun et al., 2014) also presented optimum activity at 60°C.

According to Dybowska and Fujio (1996) at high values of temperature, the process of coagulation is decreased. The increase in milk temperature affects the protein aggregation, increase the velocity of gel formation (McMahon et al., 1984) and cause the shortening of the protein matrix due the raise of hydrophobic interactions (Ahmed and Helmy, 2012). The main factor responsible for the increase of the heated milk coagulation time is the complex formed between κ-casein and β-lactoglobulin or α-lactalbumin although the equilibrium alterations of the salt are also involved (Balcones et al., 1996).

Figure 4 shows the stability of milk-clotting enzymes from *P. albidus* at different pH values. The enzymes presented activity in all study conditions, however, a reduced stability was observed with the increase of pH values. The highest milk-clotting activities were observed at pH 4.0. At pH 5.0 and 6.0, the enzymes maintained 51.34 and 30.82% of activity, respectively. The highest

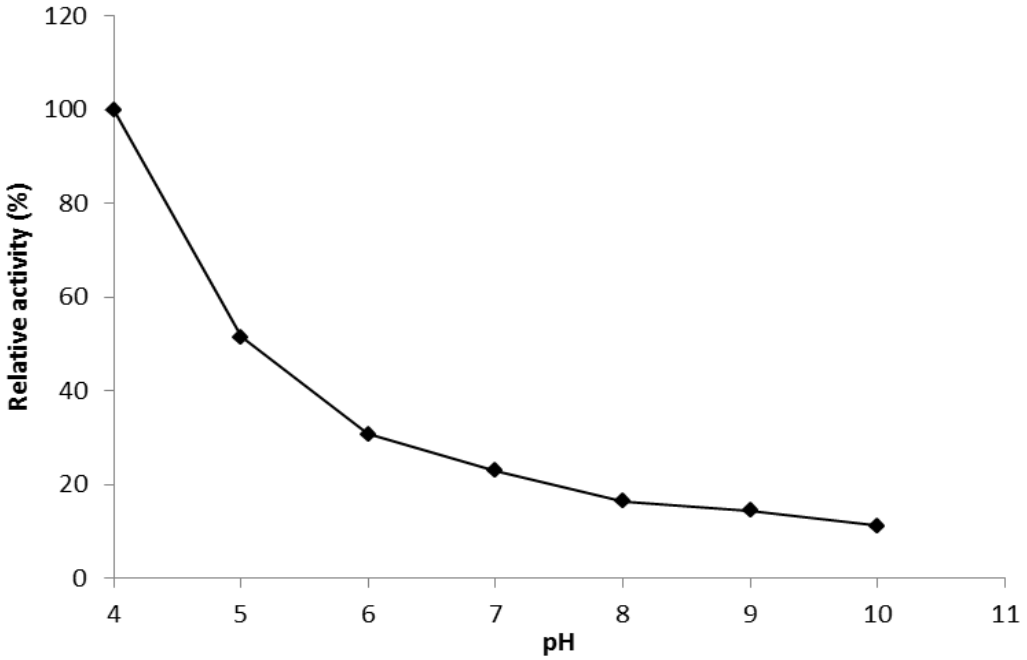

Figure 4. Effect of pH on the stability of milk-clotting enzymes from *P. albidus* crude extract, after 1 h of incubation.

loss of activity was observed at pH 10.0 present only 11.21% of activity. Merheb-Dini et al. (2010) reported similar results using milk-clotting enzymes from *T. indicae-seudaticae* N31. In this study, the highest milk-clotting activity were determined at pH 3.5. Yegin et al. (2012) reported enzymatic stability of *Mucor mucedo* DSM 809 in the pH range from 5.0 to 5.5 with loss of milk-clotting activity at pH higher than 6.0. The different levels of resistance in thermal treatment by milk-clotting enzymes vary according to the protein origin (Hayaloglu et al., 2014). Figure 5 shows the crude extract enzyme stability to different values of temperature. After 1 h of incubation at 40°C, the enzyme presented the highest milk-clotting activity, but at 50°C there was decrease to 34%. The enzymes lost 72.71, 75.85 and 78.80% of activity at 60, 70 and 80°C, respectively. *T. clypeatus* MTCC 5091 was stable between 35 and 50°C, retaining more than 80% of the activity, with a fast decrease according to the increase of temperature (Majunder et al., 2015). In the study of Alecrim et al. (2015) the enzymes from *A. flavo furcatis* presented thermal stability higher than 70% between 40 and 60°C, however in the highest temperature was observed a decrease to 28% of activity.

The effect of metallic ions and inhibitors in the milk-clotting enzymes activity are shown in Table 1. The ion Zn^{2+} raised the milk-clotting activity in 78%. The ions Mn^{2+}, Fe^{2+} and Cu^{2+} caused a reduction of 97.60, 78.75 and 35%, respectively. Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} promoted a complete inhibition of the milk-clotting enzymes (Figure 5). Sun et al. (2014) showed that Zn^{2+} ions raised the milk-clotting enzyme activity from *R. microsporus* var. *rhizopodiformis*. Ahmed and Helmy (2012) also observed the raise of activity in the presence of Zn^{2+} ions (67.7%) in the study with *Bacillus licheniformis* 5A5. Cu^{2+} and Mg^{2+} ions stimulated milk-clotting enzymes from *P. oxalicum* in the report of Hashem (2000). According to Merheb-Dini et al. (2009) the ions can link in amino acid residues and modify the protein structure which can have positive or negative influence in the proteolytic activity.

In the tests using different inhibitors, the results showed the presence of different milk-clotting protease groups from *P. albidus* with the highest prevalence of cysteine-proteases. Milk-clotting proteases from *P. albidus* maintained stability with EDTA. They retained 93.7% of milk-clotting activity. Pepstatin A and PMSF caused inhibition of 35 and 49.5%, respectively. Iodoacetic acid caused the highest inhibition of milk-clotting proteases (37.7%). These results demonstrated that SH groups probably can be involved in the catalytic mechanism and it is suggested that milk-clotting enzymes from *P. albidus* belong to cysteine protease family.

After 72 h of treatment, MRC5 cell viability was equal or superior to 100% and the cells did not present any morphological changes when compared with negative control (Dimethyl Sulfoxide [DMSO]). The positive control (doxorubicin) presented 23% of cell viability when tested with 20 µM or 34 µg/ml (Figure 6). These results show that the extract from *P. albidus* does not presenttoxicity and can be used with safety in food industry. Al-Temimay et al. (2015) and Lai et al. (2013)

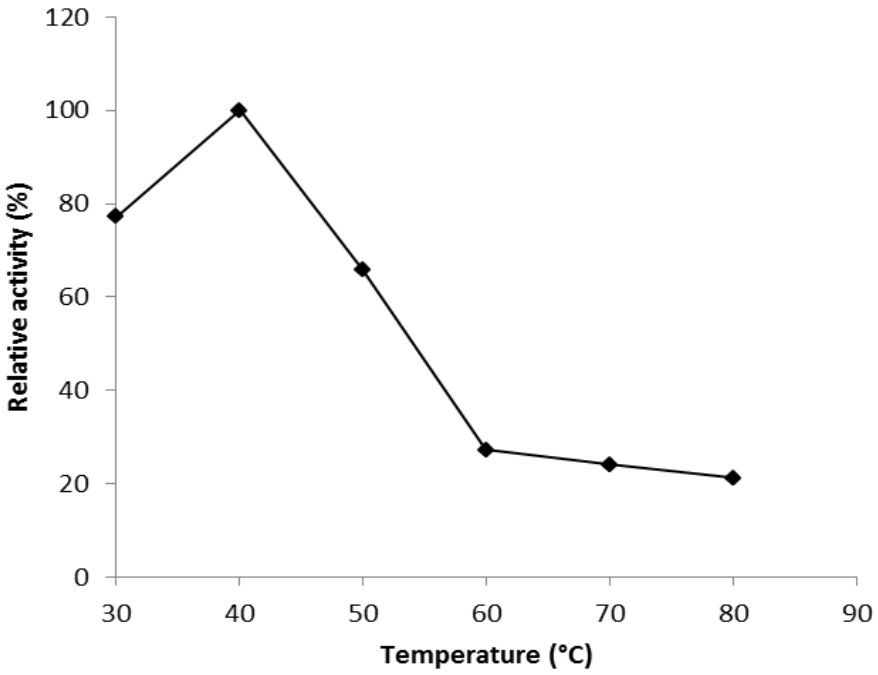

Figure 5. Effect of temperature on the stability of milk-clotting enzymes from *P. albidus* crude extract, after 1 h of incubation.

Table 1. Effect of ions and inhibitors in milk-clotting activity of *P. albidus* milk-clotting enzymes.

Chemicals	Concentration (mM)	Relative activity (%)
Control	—	100 ± 0.00
Zn^{2+}	10	178 ± 0.20
Mn^{2+}	10	98 ± 0.17
Fe^{2+}	10	79 ± 0.04
Cu^{2+}	10	35 ± 0.02
Mg^{2+}	10	0 ± 0.00
Na^{+}	10	0 ± 0.00
Ca^{2+}	10	0 ± 0.00
K^{+}	10	0 ± 0.00
EDTA	10	94 ± 0.13
Pepstatin	1	65 ± 0.22
PMSF	10	51 ± 0.29
Iodoacetic acid	10	38 ± 0.20

reported that the extracts from *Pleurotus ostreatus* and *Hericium erinaceus* did not present toxicity effect against MRC5 fibroblasts strains. The aqueous extract from *Lignosus rhinocerotis* was also not toxic to MRC5 cells (Lau et al., 2013).

Conclusions

From the five edible mushrooms evaluated, *P. albidus*, a species nontoxic, from Amazonic ecosystem, produces milk-clotting enzymes. This potential coagulant expresses optimum activity at 60°C, pH 6 with higher stability at 40°C, pH 4, with the presence of cysteine proteases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

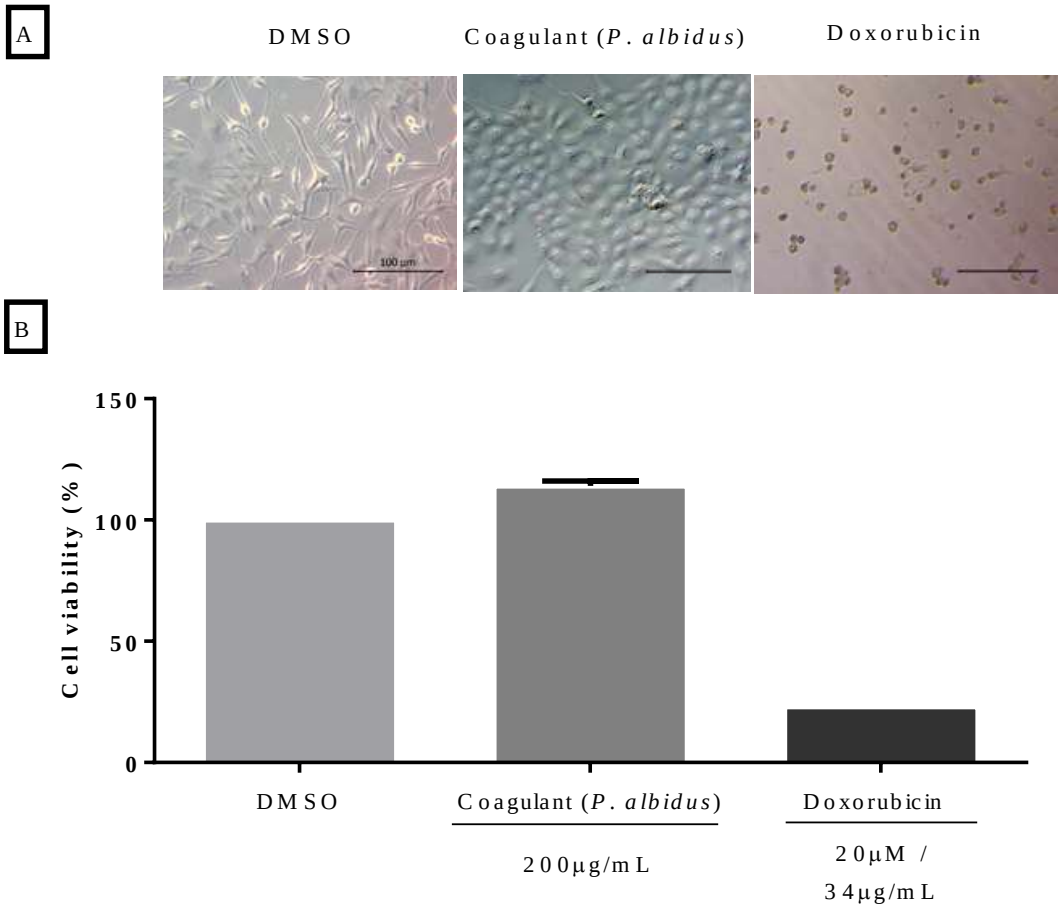

Figure 6. Result of cytotoxic assay of *P. albidus* coagulant extract against human fibroblasts MRC5 after 72 h of treatment. A: MRC5 cells after treatment with DMSO, *P. albidus* coagulant extract, Doxorubicin. B: DMSO (negative control), cell viability of *P. albidus* extract, 0.01% (w/v) and Doxorubicin (positive control -20 µM or 34 µg/mL).

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