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Use of Vero cell line to verify the biodetoxification efficiency of castor bean waste

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ABSTRACT

Ricin is a toxic protein present in castor bean seeds (*Ricinus communis*). A toxic residue named castor bean waste is generated during biodiesel production process, such as that developed by PETROBRAS (the national petroleum company of Brazil). Solid-state fermentation (SSF) was used to detoxify castor bean waste through the *Penicillium simplicissimum* growth. After 24 h of fungal growth, the ricin was no longer identified by Sephadex G-50 gel chromatography. In order to verify the biological activity of ricin after several treatment stages, an *in vitro* assay using Vero cell line was carried out. Through this methodology, it was verified that after 24 and 48 h of treatment, the cell culture showed slightly growth inhibition. The waste was completely detoxified only after 72 h of fungal growth. This fact shows that an *in vitro* assay is important to verify the real efficiency of detoxification. Moreover, a relationship between the fungal protease production and the waste detoxification was observed.

1. Introduction

Ricin is a protein present in castor bean (*Ricinus communis*) seeds, being primarily responsible for the toxicity of castor seed and the cake originated from its oil extraction. It is known as the one of the most toxic proteins. Ricin performs its mechanism of toxicity through the inhibition of protein synthesis in cells, being classified as a ribosome inactivating protein (RIP) [1]. It is a type 2 RIP (RIP-2), with a molecular mass of approximately 66 kDa, composed by two chains of polypeptide – A chain (RTA) and B chain (RTB) with approximately 32 kDa and 34 kDa, respectively – linked by a disulfide bond [2,3]. RTB is a lectin and binds itself to the cell surface through galactose residues present on cytoplasmatic membrane, enabling the endocytosis of the toxin. The internalized ricin is separated into its subunits if reaches the Golgi apparatus, and then it is transported to the endoplasmic reticulum. Subsequently, the RTA is transported to the cytoplasm, where it contacts with the ribosome. The RTA is responsible for the inactivation of ribosomes in cells, removing an adenine present in the conserved 28S ribosomal RNA loop, which belongs to the 60S ribosome subunit. As this loop is involved in binding to elongation factors of protein, the modified ribosomes are thus unable for further protein synthesis, leading to cell death [2,4].

The castor bean cake is the most important and traditional by-product of the castor oil production. It is a waste very rich in nutrients and organic matter and is often used as fertilizer [5]. The cake is also a good candidate to be used in animal feed composition. However, the presence of toxic compounds limits its use for these purposes [6,7]. The PETROBRAS Research Center (Cenpes) developed a process to obtain biodiesel from castor bean seeds [8]. The castor bean seeds are grounded and added directly to the chemical reactor in the presence of ethanol and an alkaline catalyst and, after the transesterification reaction, a residue named castor bean waste is produced. This residue is extremely alkaline, has no value after the process of biodiesel production and, unlike the castor bean cake – obtained from the pressing of castor bean seeds – cannot be used as fertilizer.

An alternative for this agro-industrial residue would be to use it as a culture medium for solid-state fermentation (SSF) to obtain biotechnological products [7,9,10]. SSF is a fermentation process which uses a solid culture medium as source of nutrients and support for the growth of microorganisms. The water content in the medium should be the minimum necessary to ensure appropriate growth and metabolism of cells, but not exceeding the

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maximum capacity of water retention in the matrix [11,12]. Besides being a process for bioproducts obtainment, the SSF is a promising process for removing toxic compounds from agro-industrial residues [7,9,13–15]. Our group has developed a new low-cost process for biodetoxification of castor bean waste through solid-state fermentation, obtaining excellent results and, simultaneously, adding value to the residue through the production of a biocatalyst [7,15]. Based on this work, another research group has used castor bean residue as solid medium in SSF to obtain enzymes (tannase and phytase) from *Paecilomyces variotii* and simultaneously detoxify the residue obtaining good results [9].

Several other attempts to detoxify castor bean cake have been carried out [16–19]. Some of these methods showed efficacy in the treatment, eliminating the ricin, but without consider the economic viability of processes for large-scale application. Almost all the works about castor bean detoxification are based on detection of ricin by classical methods such as SDS-PAGE, ELISA and Western-blot. However, these traditional methods do not take into account the biological activity of ricin. This toxic protein may be present in the waste but in an inactivated form, or it cannot be detected by conventional methods and still be present in low concentration. Due to the high toxicity of ricin it is necessary to use an *in vitro* or *in vivo* assay that enables the analysis of its biological activity. Since the use of live animals for routine analysis of toxicity is increasingly out of favor for ethical reasons, one alternative would be an *in vitro* assay based on cultured animal cell. Animal cell cultivation is widely used as model for analysis of toxins [9,20–23]. Madeira Jr et al. [9] used RAW cells to attest the detoxification process by *P. variotii*, reporting nearly 100% of efficiency after 72 h of fermentation with a 10 μg of protein/ml extract. Although, there are many strategies described to detoxify castor bean residues (physical, chemical and biologic) few taking into account its final biological activity using *in vivo* or *in vitro* analysis [9]. In the present study, it was used a culture of Vero cell line for the toxicity assay to verify the real efficiency of the detoxification process. This is the first study that reports the use of a biological test in Vero cells and validates the efficiency through the analysis of a dose–response curve of toxicity in autoclaved, fermented and *in natura* extracts. The biological detoxification was carried out through *Penicillium simplicissimum* growth by solid-state fermentation using castor bean waste as culture medium. In addition, it was analyzed the possible relationship between the elimination of toxicity and the protease production by the fungus.

2. Materials and methods

2.1. Microorganism and propagation

The strain of *P. simplicissimum* used in this work was isolated by Freire [24], selected as a good lipase producer by Gutarra et al. [25,26] and pointed as a fungal strain able to detoxify washed castor bean waste by solid-state fermentation by Godoy et al. [7]. To obtain spores, the fungal strain was propagated at 30 °C for 7 days in a medium with the following composition (%, w/v): 2.0 soluble starch; 0.025 $MgSO_4{\cdot}7H_2O$; 0.05 KH_2PO_4; 0.5 $CaCO_3$; 0.1 yeast extract; 1.0 olive oil; and 1.0 agar. The spores were suspended in 100 mM phosphate buffer (pH 7) and counted in a cell-counting chamber.

2.2. Solid-state fermentation (SSF)

Castor bean waste, a solid biodiesel production residue [9], was used as solid-state fermentation culture medium. The waste was ground in a laboratory knife mill and separated in a sieve shaker using a sieve presenting mesh tyler equal to 14. The final particle sizes of the waste are, then, smaller than 1.18 mm of equivalent diameter. The castor bean medium was autoclaved (121 °C; 1 atm; 15 min) and moisturized to 48.5% (w/w). The fermentations were carried out in lab-scale, tray-type bioreactors containing 20 g of waste, forming a 1 cm-deep layer in the bioreactor. The medium was inoculated with 10^7 spores/g of dry solid and incubated in a chamber with conditions set to 30 °C and 95% water saturation. Fermentation samples (whole trays) were removed at 24 h intervals for up to 120 h.

2.3. Enzyme extraction

After the fermentation times, 100 mM phosphate buffer (pH 7.0, 5 ml/g) was added to each tray containing the fermented solids, and enzyme extraction was performed in a rotary shaker at 35 °C and 200 rpm for 20 min. Afterwards, a solid–liquid separation was obtained by pressing followed by centrifugation at 2000 × *g* for 5 min [27]. The pressed solids were retained for protein extraction in order to determine their toxic composition after SSF. The supernatant, which presents no toxin (data not shown), was stored at −20 °C and used to determine protease activity.

2.4. Protease activity

Protease activity was determined by the method described by Charney and Tomarelli [28], based on the formation of stained proteins derived from the digestion of azocasein solution precipitated with trichloroacetic acid (TCA). The absorbance of this solution was then determined in a spectrophotometer at 428 nm. One unit of protease activity (U) was defined as the amount of enzyme causing an absorbance unit difference between the sample and its corresponding control per minute under the described assay conditions. Enzyme activity was expressed as units per gram of the initial dry solid medium.

2.5. Glucosamine determination

Biomass quantification was done indirectly through the determination of the N-acetylglucosamine content in the fungal biomass, according to the method described by Aidoo et al. [29]. Thus, biomass concentration was indirectly expressed as mg of N-acetylglucosamine per gram of dry castor bean waste (mg/g).

2.6. Protein extraction

Proteins were extracted from *in natura*, autoclaved and fermented castor bean waste, for toxicity assays. Castor bean waste was ground into a fine powder and proteins were extracted by shaking the contents with 100 mM phosphate buffer saline (PBS), pH 7.0, in the proportion 1:4 (w/v). The mixture was kept stirring vigorously for 3 h at room temperature and then centrifuged at 7900 × *g*-force for 15 min. The supernatant was used for ricin detection and cytotoxicity assays, as described in the item 2.9. The protein determination in the extracts was done according to the method of Bradford [30], and ovalbumin (1 mg/ml) was used as a standard protein for the calibration curve.

2.7. Fractionation of protein extract

For ricin separation, the supernatant obtained after protein extraction (500 μl) was loaded in a Sephadex G-50 column, pre-equilibrated with trifluoracetic acid (0.1%, v/v), at a flow rate of 1.0 ml/min.

2.8. Cell culture

Vero cells (African Green Monkey kidney cells) were cultivated in culture flasks (TPT-90025). Cells were grown at 37 °C in Dulbecco's Modified Eagle Medium (DMEM/Sigma-D1152) supplemented with fetal bovine serum 10% and 1× penicillin–streptomycin (Sigma-T4799).

Cells were detached from the flasks by treatment with trypsin (0.5 mg/ml)/EDTA (0.2 mg/ml) in PBS for 5 min at 37 °C. Detached cells were suspended in the growth medium and incubated in culture flasks at density of 2.0×10^4 cells/ml in each flask. This culture was maintained at 37 °C for 48 h.

2.9. Cytotoxicity assay

Protein pool from *in natura* (positive control), autoclaved and fermented (24, 48, 72 and 120 h) castor bean waste were previously sterilized in 0.22 μm pore filter (TPP-99722). Filtered samples were then incubated with cells at a concentration of 10 μg/ml. Initial cell concentration was 1.6×10^5 cells per flask. PBS pH 7.0 was used as negative control. Cell counting at 0, 12, 24 and 36 h were carried out in 3 different fields using an optical microscopy (40× magnification) to observe the toxic effect. A conversion factor (×225) was used to determine the cell concentration in each sampled plate well.

The apparent specific growth rate (μ) was estimated using the period 0–24 h (in which the cultures are in exponential phase) using the following equation [31]:

$$\mu = \frac{\Delta \ln(n_v)}{\Delta t} \quad (1)$$

where n_v is the viable cell concentration (cells ml^{-1}) and t is the growth time (h).

A lactate dehydrogenase (LDH) activity assay was also carried out, in order to confirm the cytotoxicity results. Cell death was measured using the Cytotoxic Detection Kit (Roche) at 24 and 48 h after incubation. The cell culture supernatant (100 μl) was placed in each well (triplicate for each sample) of a 96-well plate, followed by 100 μl LDH detection solution, and incubated in the absence of light for 30 min at room temperature. The colorimetric range was measured with a Thermo plate

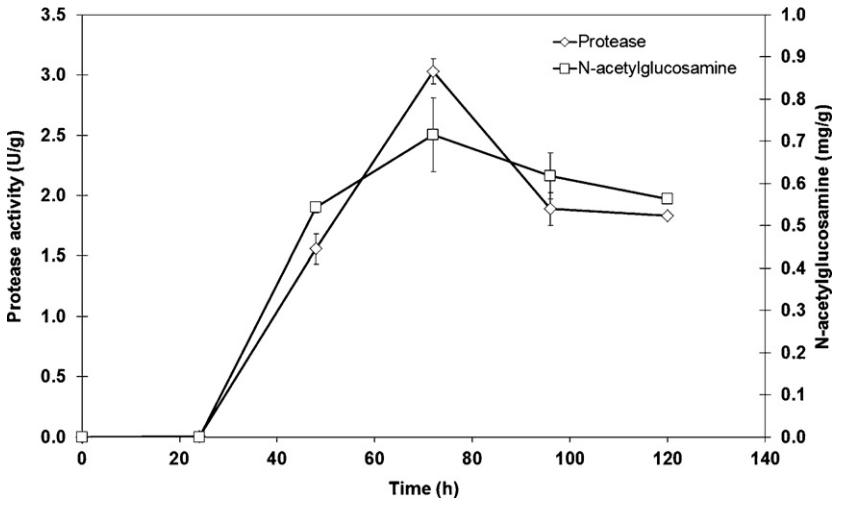

Fig. 1. *P. simplicissimum* growth and its protease production profiles in solid-state fermentation using castor bean waste as culture medium.

reader at 492 nm. Cytotoxicity was calculated following the instructions provided by the kit manufacturer.

In parallel, a dose–response curve of toxicity was carried out, using different protein concentrations of the extracts: *in natura* waste (5 μg/ml, 1 μg/ml, 100 ng/ml, 10 ng/ml and 1 ng/ml); autoclaved waste (100 μg/ml, 50 μg/ml and 200 μg/ml) and 72 h fermented waste (100 μg/ml, 50 μg/ml and 200 μg/ml).

All experiments of cytotoxicity assay were carried out in triplicate.

3. Results and discussion

The fungus *P. simplicissimum* grew satisfactorily in the toxic castor bean waste reaching a maximum at 72 h (Fig. 1), which was expressed indirectly by the N-acetylglucosamine content. Since the fungus was able to grow in this unfriendly residue, the search for toxic components at different fermentation stages was carried out. The protein extracts, obtained from *in natura* and fermented castor bean waste, were loaded in a Sephadex G-50 column (molecular exclusion chromatography) to verify the presence of ricin in the non-fermented waste (*in natura*) and after several fermentation times. The results confirm the effectiveness of treatment (Fig. 2). Two peaks are observed majority after elution of the crude extracts, being the first one identified as the fraction containing ricin [32]. This peak is evident in the sample of the *in natura* castor bean waste, with a subsequent decrease or total disappearance in the fermented samples (Fig. 2). The second peak is probably due to the presence of other proteins produced by the fungus and also the ones present in the castor bean waste.

Ricin is a strongly toxic protein and it is known that a single molecule in the cytoplasm is able to stop protein synthesis, through inactivation of approximately 1500 ribosomes per minute [2]. Even after the detection of ricin in the samples, an assay to verify if the detected ricin presents biological activity or not is necessary. Furthermore, it is necessary to be sure if the non-detection of ricin, due to a possible low sensitivity of the used method, really generates a non-toxic and safe product. Thus, it is necessary to evaluate the toxicity of the samples in a living system, validating the efficiency of the treatment. The proteins extracted from *in natura*, autoclaved and fermented castor bean waste were placed in contact with Vero cell line. Animal cells are widely used as a model for analysis of *in vitro* toxicity of several compounds [20–23].

The extract from the autoclaved castor bean waste as well as the *in natura* one led to cell death, with total elimination after 36 h (Fig. 3). The extracts from 24 h to 48 h fermented castor bean waste showed slightly cell growth inhibition, but do not led to cell death. At these fermentation times the cell growth rates (μ) were about 50% of the ones reached in the control, in which sterile PBS was added instead of extract from castor bean waste (Table 1). However, the extract from the fermented samples after 72 h did not show any damage to cell growth, which behaved very similar to negative control (Fig. 3, Table 1). This fact can be evidenced in the micrographies shown in Fig. 4 and in LDH activity assays (Fig. 5). During *in vitro* cultivation, the Vero cells grew adhered onto the surface of cell culture flask forming a monolayer culture. When the cells begin to die, they are detached from the flask surface, which can be observed by light microscopy (Fig. 4). The cells grown in the presence of 72 h fermented extract (Fig. 4J–L) have a growth similar to the negative control (Fig. 4A–C). These data confirm the efficiency of castor bean waste detoxification by *P. simplicissimum* fungus through solid-state fermentation. In order to confirm the total elimination of the toxicity, an enzyme activity assay was also carried out based on the release of lactate dehydrogenase (LDH) by the dead cells. LDH activity was not observed when Vero cells were exposed to the 72 h and 120 h fermented extracts (Fig. 5), confirming the efficiency of the treatment. Interestingly, although the ricin in autoclaved solids was strongly reduced (Fig. 2), it is still active in the solid waste, resulting in cell death such as in the non-fermented waste (Figs. 3 and 4). The least amount of ricin in autoclaved castor bean waste may be due to factors of the autoclaving process, which could be decreasing the proteins solubility, thus resulting in greater difficulty of detection by molecular exclusion chromatography. In the cells exposed to *in natura* extracts (Fig. 4D–F), the beginning of the cell death in 12 h of exposure is clear, while in the cells exposed to autoclaved sample (Fig. 4G–I), the cell death occurs in 36 h of exposure. The autoclaving process leads to later cell death, but leaves the castor bean waste still toxic. This reduction of toxicity by autoclaving also occurs in the other fermented samples, since the waste is autoclaved prior to microorganism inoculation. However, this reduction is small and there is no complete inactivation

Table 1
Apparent specific growth rate (μ) of Vero cells in the presence of different extracts.

Sample	μ (h^{-1})
Control	0.041
In natura	–
Autoclaved	–
Ferm. 24 h	0.022
Ferm. 48 h	0.021
Ferm. 72 h	0.036
Ferm. 120 h	0.046

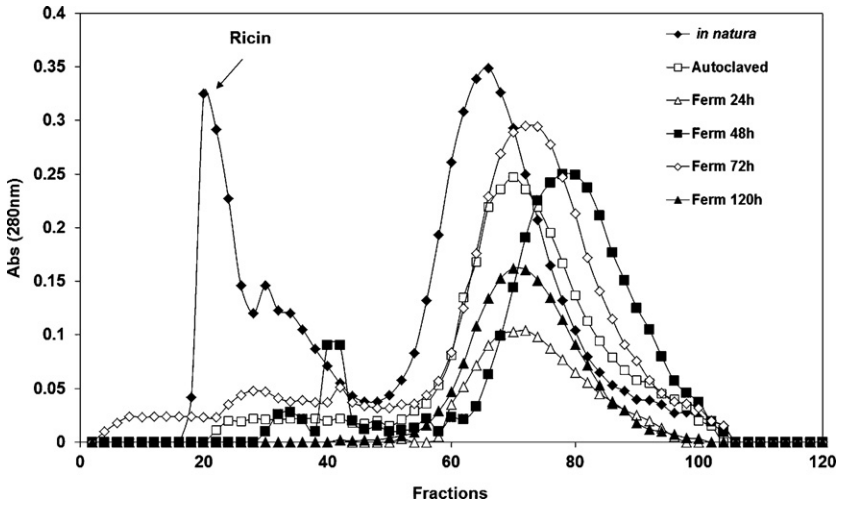

Fig. 2. Chromatography of extracts from castor bean waste: in natura, autoclaved and after different fermentation time. The extracts were loaded on Sephadex G-50 (30.0 cm × 1.5 cm) and eluted with trifluoracetic acid, at a flow rate of 1.0 ml min^{-1}. The fractions were monitored at 280 nm.

of the toxin. The combination of this autoclaving step followed by SSF process leads to a total elimination of the toxicity of the waste (Fig. 5).

The detoxification of castor bean residues by solid-state fermentation has been described in other works. Our group used the biological detoxification through SSF and reached a total elimination of ricin in the washed castor bean waste using the same fungus strain, with simultaneous lipase production [7]. The authors used a castor bean waste pre-treated with HCl – in order to decrease the pH of the medium – reducing the ricin contents by SSF to non-detectable levels, analyzed by SDS-PAGE and molecular exclusion chromatography. In the present work, a biodetoxification of a non-treated castor bean waste was carried out – which reduces the stages of the treatment and also the liquid residue generation. Furthermore, biological activity was assayed *in vitro* to confirm the biodetoxification process efficiency. Madeira et al. [9] also used animal cells to demonstrate the biodetoxification of castor bean residues, reaching about 100% of efficiency with the residues fermented for 72 h by *Paecilomyces variotii*. However, the authors used only the minimum inhibitory concentration of the control and fermented extracts (1 μg/ml), making it impossible to verify the true efficiency or robustness of the process. Several other attempts for thermal and chemical detoxification of castor bean residues have been made, but often without taking into account the cost of the process, besides it was not carried out any biological activity to confirm the efficiency of the process [16–18]. As observed in this study, even apparently detoxified samples (Fig. 2) – analyzed by conventional methods of ricin detection – can also present a high toxicity as the ones in the autoclaved sample (Fig. 3). Moreover, the *in vitro* assay is very important to check whether the ricin detected in the samples still shows biological activity, avoiding misinterpretation of results.

In order to determine the real efficiency of the process, a dose–response curve of toxicity was carried out using the *in natura,* autoclaved and 72 h fermented waste at different concentrations (Fig. 6). Lower protein concentrations in the *in natura* extract were used to verify the minimum dose in which is possible to observe the growth inhibition. On the other hand, concentrations above 10 μg/ml were used for the autoclaved and fermented extracts in order to verify if the toxin was actually eliminated. The *in natura* extract with 10 ng/ml still has considerable toxicity, while the 1 ng/ml sample does not present such high toxicity (Fig. 6).

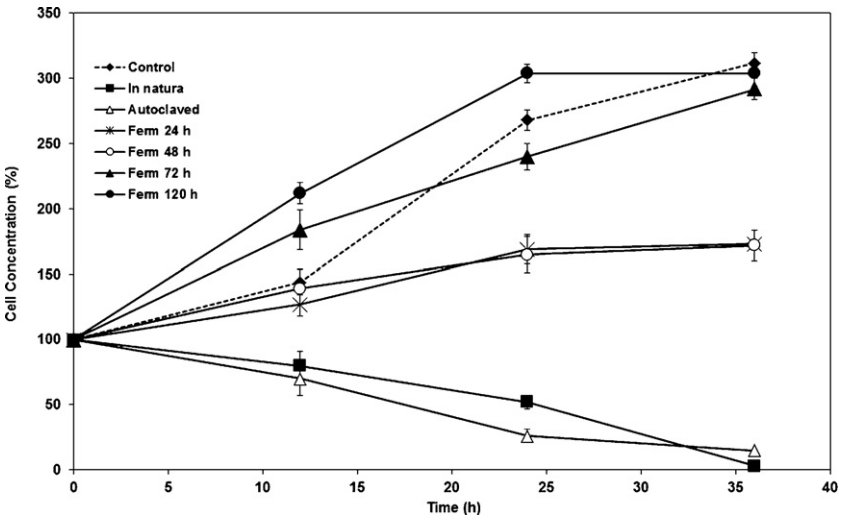

Fig. 3. Vero cells growth in the presence of different extracts from castor bean waste (treated and non-treated). Ten micrograms of protein were applied in the cytotoxicity assay. The experiments were carried out in triplicate.

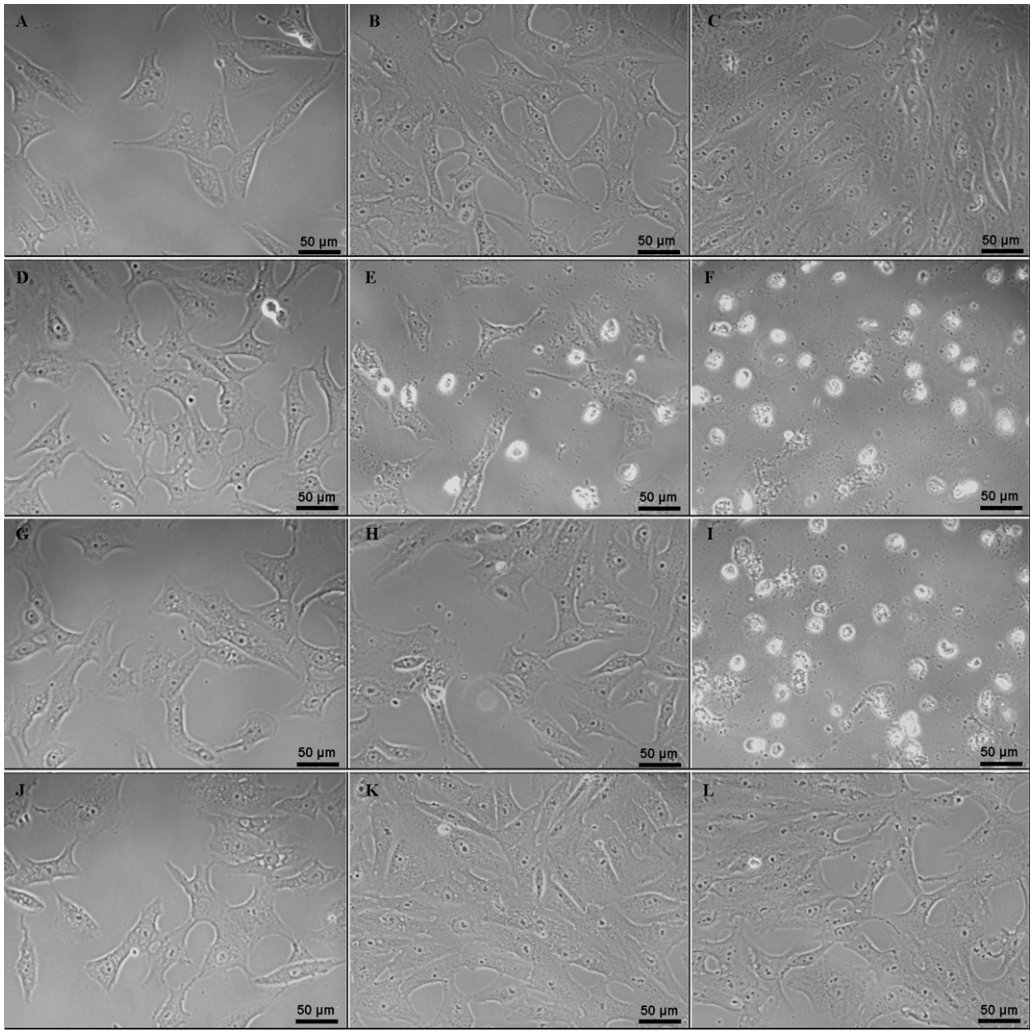

Fig. 4. Micrographs of Vero cell culture under different conditions. A–C: cell culture control at 0, 12 and 36 h of growth, respectively; D–F: cell growth at 0 h, 12 and 36 h, respectively, in the presence of extract from *in natura* castor bean waste; G–I: cell growth at 0 h, 12 and 36 h, respectively, in the presence of extract from autoclaved castor bean waste; J–L: cell growth at 0, 12 and 36 h, respectively, in the presence of extract from 72 h castor bean waste fermented.

However, all fermented samples showed zero toxicity at concentrations up to 10,000 times higher than *in natura* extract. These results confirm the real efficiency of the process used to eliminate the toxicity present in the castor bean waste.

The reduction of ricin amount achieved in the treatment of castor bean waste through solid-state fermentation is probably related to the fungal growth and the enzyme production (Fig. 1). The maximum *P. simplicissimum* growth and the protease production were

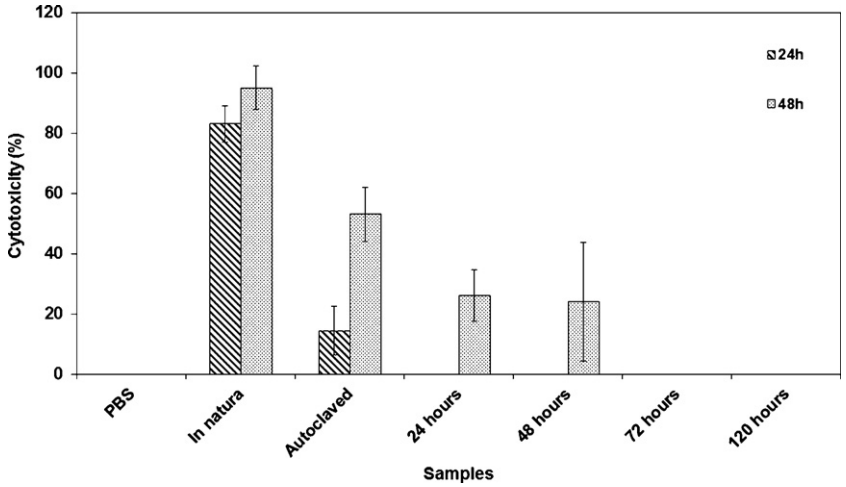

Fig. 5. Lactate dehydrogenase (LDH) released by Vero cells incubated for 24 h and 48 h with castor bean waste: *in natura*, autoclaved and after 24, 48, 72 and 120 h of fermentation (protein concentration = 10 μg/ml). PBS was used as control. The experiments were carried out in triplicate.

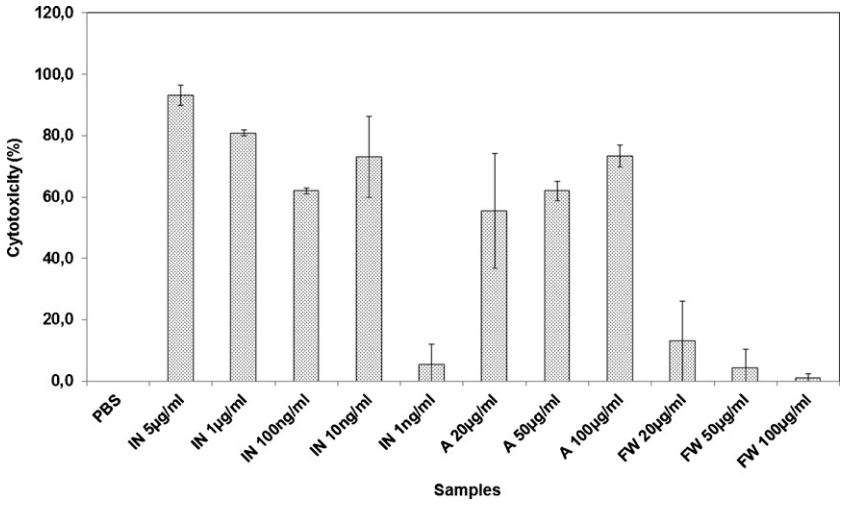

Fig. 6. Toxicity dose–response curve: lactate dehydrogenase (LDH) released by Vero cells incubated for 48 h with different concentrations of: *in natura* (IN), autoclaved (A) and 72 h fermented (FW) castor bean waste. PBS was used as control. The experiments were carried out in triplicate.

both reached after 72 h of fermentation. At this time, the fermented solids did not present biological activity in Vero cells, suggesting that the elimination of ricin from castor bean waste is closely linked to the production of protease by the fungus.

4. Conclusions

The fungus *P. simplicissimum* grew in the residue of castor bean and eliminated the ricin totally even when fermented castor bean residue extracts with high protein concentration were analyzed. The animal cell assay confirms the toxin elimination present in the castor bean waste, making it safe for future technological applications. Moreover, this study shows how important is to carry out an *in vitro* assay to demonstrate the efficiency of the detoxification process, avoiding misinterpretations. Oftentimes ricin cannot be detected by conventional methods of toxic proteins detection, but it is still present and has harmful effects to cells, as showed in this study. In other cases, the ricin could be detected by other methods, but showed no biological activity. Thus, in order to have reliable results it is recommended the use of animal cell as routine toxicity assay, verifying the efficiency of the detoxification process.

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