International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 5 (2014) pp. 810-823**
http://www.ijcmas.com

Original Research Article

Concomitant Production of Peptidases and Lipases by Fungus Using Agro-industrial Residue in Solid-state Fermentation

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A B S T R A C T

Agro-industrial waste, such as wheat bran (WB) and cottonseed meal (CM), are a rich source of nutrients with low cost. In order to achieve the best production of peptidases and lipases by the filamentous fungus *Fusarium oxysporum*, these residues, in different proportions, were analyzed as culture medium for solid-state fermentation. Biochemical characterization of the enzymatic extract was also studied. The use of WB alone demonstrated a higher enzyme production (228.88 U/mL) when compared with the different media combinations. The fungus concomitantly produced serine peptidase and lipase, both alkaline, with high stability over a wide pH range and some stability at different temperatures. For peptidase there was a strong lost of activity against the surfactant SDS, whereas lipase demonstrated high activity (156%) against CTAB. The possibility of industrial adaptation of the bioprocess and the prospect of commercial application of the produced enzymes, such as detergents, leather industry, agrochemicals, biopolymers and biodiesel, confirms that this is a valuable alternative to obtain biotechnological products with high added value at an affordable cost, while helping to solve environmental problems.

Keywords

Agro-industrial waste, Fusarium oxysporum, lipase, peptidase, solid state fermentation.

Introduction

Enzymes are one of the most important groups of biological products and find several usages once they can catalyze reactions such as hydrolysis, polymerization, transfer of functional groups, redox, dehydration and isomerization (Namaldi et al., 2006). Freedonia Group indicates that the global market for enzymes generates a billing of around 5.8 billion dollars per year, with a forecast growth of 6.8% per year (http://www.freedoniagroup.com/DocumentDetails.aspx?ReferrerId=FG-01&studyid=2824). From industrial enzymes, approximately 75% are hydrolytic and among them stand out peptidases (EC 3.4) and lipases (EC 3.1.1.3), which represent two of the largest

groups with industrial application (Rao et al., 1998; Shu et al. 2010).

In the production of enzymes, microorganisms are an attractive option due to their growth in large quantities with a small space needed, in relatively short time, with ease of maintenance, low cost, and with the possibility of genetic manipulation. In addition, enzyme production is not associated with seasonal or geographical conditions (Rao et al., 1998; Demain, 2007).

To obtain enzymes of microbial origin on a large scale, it is used industrial fermentation (Mitchell et al., 2006). To fungi cultivation, solid-state fermentation (SSF) is the best choice because it provides fermentation conditions similar to the natural environment of the microorganism (Treichel et al., 2010). The SSF involves the microbial growth on the surface and/or inside of wet solid particles with a minimum free water, which generates a low volume of effluents. Although synthetic culture media is largely used, the possibility of using agro-industrial residues as support and source of nutrients is a low coast alternative that can also help solving environmental problems (Mitchell et al., 2006; Treichel et al., 2010).

Species of the genus *Fusarium* are widely distributed in the soil, aerial and subterranean parts of plants and in other organic materials, showing a great ability to grow on different substrates (Nelson et al., 1994). Thus, *Fusarium oxysporum* becomes an interesting candidate for production of lipolytic and proteolytic enzymes with possible industrial use.
Thereby, the aim of this study was to investigate fermentative parameters for production of peptidases and lipases by the fungus *F. oxysporum*, using agro-industrial residues as culture medium and realize biochemical characterization of the enzymatic extract to evaluate its biotechnological potential.

Materials and Methods

Chemicals

Chemicals were obtained from Sigma-Aldrich (Germany) unless otherwise indicated.

Inoculum

The used fungus belongs to the mycology collection of *Laboratório de Tecnologia Enzimática da Faculdade de Ciências Farmacêuticas de Ribeirão Preto* and was identified as *F. oxysporum*, based on its morphology and biochemical tests.

For inoculum preparation, the fungus was grown in inclined Erlenmeyer flasks of 250 mL, containing 30 mL of PDA, with 7 days incubation at 30°C. The suspension of mycelia was obtained by adding to each flask 20 mL of sterilized saline solution (0.1% ammonia sulfate, 0.1% magnesium sulfate heptahydrate and 0.1% ammonia nitrate) followed by scraping of the content with a spatula (Merheb-Dini et al., 2009).

Solid-state fermentation (SFF)

Adapting methods of Lotong and Suwanarit (1983) and Merheb-Dini et al. (2009), it was used polypropylene bags with dimension of 12 x 20 cm as containers for fermentation.

The bags were filled with 5 g of agro-industrial residue, which were WB and/or CM in different proportions (1:0, 0:1, 1:1, 3:1, and 1:3) and 9 mL of saline solution.

The containers were autoclaved and after reaching room temperature it was inoculated 1 mL of the inoculum into each bag. The bags were incubated at 30°C, for 24 to 168 h. After every 24 h a bag was collected and to it was added 40 mL of distilled water at 4°C. The material was homogenized, filtered and centrifuged at 5,000 *g* (4°C, 20 min). The supernatant, called fermentation extract (FE) was collected for quantification of enzymatic activities.

Profile of enzymes produced by SFF

Determination of proteolytic activity of the FE with casein

For all variations of culture medium, proteolytic activity was determined according to Sarath et al. (2001) with modifications. The reaction mixture was composed of 200 μL of FE; 100 μL of NaH_2PO_4 buffer 0.05 mol/L (Synth, Brazil), pH 6.5 and 1000 μL of 1% casein. The reaction was conducted at 40°C (20 min) and was interrupted with 600 μL of 10% TCA (Synth, Brazil).

Reaction tubes were centrifuged at 10,000 *g* (25°C, 15 min). The absorbance of the supernatant was determined by spectrophotometry at 280 nm wavelength, against each blank. Proteolytic activity was expressed in units of activity/mL (U/mL). One unit of activity is equal to the amount of enzyme required to promote the release of 1 μmol of tyrosine/min under assay conditions (Gupta et al., 2002).

Determination of lipolytic activity of the FE

Lipase activity was determined according to Kanwar et al. (2005) with modifications. A stock solution of 0.02 mol/L of *p*-nitrophenylpalmitate was prepared in isopropanol (Synth, Brazil). For each 25 μL of stock solution, the diluted solution (DS) was obtained at 875 μL of Tris-HCl 0.05 mol/L pH 8.0.

The reaction mixture consisted of 50 μL of FE and 450 μL of DS. The reaction was conducted at 40°C (20 min) and was interrupted with 166 μL of ethanol:acetone (1:1) (both Synth, Brazil) at 4°C. Reaction tubes were centrifuged at 10,000 *g* (25°C, 1 min). The absorbance of the supernatant was determined at 410 nm, against each blank. One unit of lipolytic activity is defined as the amount of *p*-nitrophenol released by the hydrolysis of *p*-nitrophenylpalmitate by 1 μL of enzyme assay (Kanwar et al., 2005).

Fractionation by ethanol

In order to reduce the amount of impurities in the FE for biochemical characterization, it was subjected to concentration and partial purification with ethanol (92.8 GL). The best condition of precipitation was the ratio 1:2 (FE:ethanol). Fractionation was conducted at -20°C for 24 h. After being centrifuged at 5,000 *g* for 20 min, the supernatant was discarded and the pellet was resuspended in acetate buffer 0.025 mol/L, pH 5.0 in a proportion of 6 mL of buffer to 1 mL of precipitate. The resuspended extract was called enzymatic extract (EE).

Biochemical characterization of the enzymes of the EE

Determination of proteolytic activity of the EE with azocasein

The reaction mixture was composed of 100 μL of EE; 100 μL of the appropriate pH buffer, and 200 μL of 1% azocasein in the tested pH. The reaction was conducted

at 40°C (5 min) and was interrupted with 800 μL of 10% TCA. Reaction tubes were centrifuged at 10,000 *g* (25°C, 15 min). It was transferred 400 μL of the supernatant into a test tube and to it was added 466.5 μL of NaOH 1 mol/L (Synth, Brazil). The absorbance of the supernatant was determined at 440 nm against the respective blanks (Ducros et al., 2009).

Determination of lipolytic activity of the EE

The determination of lipolytic activity was performed the same way as in enzyme production profile, by replacing FE to EE.

Effect of pH on activity and stability

The optimum pH was measured by performing reactions at 40°C at different pH values, in the following 0.05 mol/L buffers: acetate, pH 4,5 - 5,0; MES, pH 5.5 - 6.5; HEPES, pH 7.0 to 8.0; Bicine, pH 8.5 to 9.0, and CAPS pH 9.5 to 10.5. For the determination of proteolytic activity was prepared a solution of 1% azocasein in each of the pH values mentioned. For determination of lipolytic activity, the same buffers above were used with the stock solution to obtain a diluted solution of *p*-nitrophenylpalmitate in buffers (DSB). The performance of the pH tests followed to determination of the enzymatic activity.

The stability was evaluated by incubating the EE to exposure buffers (0.05 mol/L) in different pH values (4.5 to 10.5, with increments of 0.5) for 24 h at room temperature. For proteolytic activity 100 μL of EE were incubated in 50 μL of exposure buffer. After exposure period, it was added 50 μL of reaction buffer (0.15 mol/L) at the optimum pH determined, 200 μL of azocasein 1% and the activity determination followed. For lipolytic activity, 50 μL of EE were incubated in 50 μL of buffer exposure and after 24 h it was added 450 μL of DSB and the reaction followed to determination of lipolytic activity.

Effect of temperature on activity and stability

Once defined the optimum pH, the optimum temperature was determined by the same reactions at temperatures from 25 to 90°C, with increments of 5°C. The thermal stability was examined by incubation of the EE in temperatures of 30, 35, 40, 45, 50, 55 and 60°C for 5, 15, 30 and 60 min. Then the enzyme activities were determinate.

Effect of inhibitors

The effect of inhibitors on the enzymatic activities was evaluated using PMSF, pepstatin, EDTA and iodoacetic acid at 0.1 mol/L (stock) (Dunn, 2001).

To each reaction tube was added EE and solutions of inhibitors, obtaining a final concentration of 0.01 mol/L. The tubes were incubated for 5 min at 40°C. Then the reagents were added and the enzyme activities were determinate.

Effect of surfactants

The effect of surfactants on the enzymatic activity, was evaluated with CTAB (Cetyl trimethylammonium bromide), SDS (Sodium dodecyl sulphate), Tween® 80 (Polysorbate 80) and Triton™X-100 (Polyethylene glycol tert-octylphenyl ether) in the concentrations of 0.5, 1.0, 1.5 and 3.0%.

For protease, it was incubated 100 μL of EE in 100 μL of the mixture of surfactant + reaction buffer and kept at 40°C (5 min).

After this time the proteolytic activity was determinate. For lipase, 50 μL of EE were incubated in 20 μL of buffer + surfactant mixture and maintained at 40°C for 5 min. Then the reaction followed to determinate lipolytic activity.

Results and Discussion

Enzyme production profile

The fungus *F. oxysporum* was inoculated in WB, CM, or different combinations of the two substrates in SSF. For both peptidase and lipase production, it was observed higher activity with WB. The highest peaks were 228.88 U/mL for proteolytic activity and 111.48 U/mL for lipolytic activity, both at 96 h (Figure 1). The combined use of substrates was not interesting, however, the use of CM alone may be useful if the goal of the process is to obtain lipase in an enzymatic extract (EE) with low proteolytic content.

Biochemical characterization

Changes in environmental conditions may affect enzymatic behavior to favor or hinder its activity temporarily or permanently. Thus, different situations were evaluated to determine the best conditions for enzyme activity (Silva et al., 2013).

Effect of pH on activity and stability

In different pH values, the enzymes showed predominantly highest activities in alkaline pH (Figure 2). The best lipolytic activity was observed at pH 8.5. The highest proteolytic activity was between pH 6.5 and 8.5, range in which there was no statistically significant difference ($P < 0.05$), indicating a wide pH range of high proteolytic activity.

Relative to proteolytic stability, after 24 h of incubation, it was observed the lower stability between pH 7.5 and 9.5 (average 88.03%), with no statistically significant difference ($P < 0.05$). Part of the pH range of optimal activity (6.5 to 8.5) is in this range. For lipolytic stability there was no statistically significant difference ($P < 0.05$) in pH range from 5.5 to 6.5 and the lowest stability was in pH 8.5, with lipase activity of 60.27%. This is precisely the pH value where there is optimum activity.

Effect of temperature on activity and stability

Regarding the effect of temperature, at 40°C, the EE presented optimum lipolytic conditions, showing a sharp drop in activity with increasing temperature (Figure 3). Lipase from *F. oxysporum* with optimal activity at temperatures near 40°C was also observed by Hoshino et al. (1992). To peptidases, temperatures between 55 and 75°C showed the highest activity with no statistically significant difference ($P < 0.05$).

To verify the effect of inactivation of the enzymes by increasing temperature, the EE was incubated at different temperatures prior to assay activity. The profile of thermal stability of peptidase (Figure 4) indicate high stability at temperatures up to 40°C, maintaining approximately 80% of residual activity after 60 min of incubation. On the other hand, for incubation at 50°C, the activity drops to 40% after 30 min.

For lipases is demonstrated greater stability at temperatures up to 40°C. After 60 min, there was approximately 60% remaining activity after incubation at 40°C and 50% activity at 45°C.

Effect of inhibitors

Study of inhibition enables to determine the class of peptidases in serine, aspartic acid, cysteine and metallo by the mechanism of catalytic action (Dunn, 2001). The results demonstrated (Figure 5) that proteolytic activity was strongly inhibited by PMSF, indicating that the peptidase produced is a serine peptidase.

Effect of surfactants

It was analyzed the enzymatic activity against four different types of surfactants: CTAB (cationic), SDS (anionic), Tween®80 and Triton™X-100 (both non ionic) (Figure 6).

At higher concentrations of surfactant, proteolytic activity was maintained at 87.02% with Tween®80 and 74.94% with Triton™X-100. For SDS, the activity was strongly lost at all concentrations, but showed a slight increase in the higher concentration. The surfactant CTAB at 0.5% retained 62.94% of activity, showing activity decrease with increasing concentration.

In experiments with lipases, the usage of Triton™X-100 remained activity around 50%, with no statistically significant difference between the concentrations analyzed ($P < 0.05$). SDS and Tween®80 caused strong lost of lipolytic activity. CTAB showed interesting results, increased activity to 156%.

This experiments have visually shown that CM could retain significantly less moisture than WB during incubation (data not shown). According to literature data, due to its physical characteristics, WB has the ability to retain high moisture. Studies on the production of peptidases in SSF, showed that the use of CM resulted in poorer performance when compared to other five industrial residues (Javed et al., 2012; Rajmalwar and Dabholkar, 2009).

Also, most researchers have used WB for the maximum production of lipases (Bhargav et al., 2008). WB is rich in carbohydrates, proteins, lipids and minerals and is easy to purchase, once the worldwide demand for wheat is very large and all countries with agricultural crops produce it abundantly (Javed et al., 2012). In biochemical characterization, concerning to effect of pH on activity and stability the results are consistent with other studies, which also demonstrated production of lipase with high activity at alkaline pH by *Fusarium globulosum* (Gulati et al., 2005) and *F. oxysporum* (Rifaat et al., 2010). Studies with *Fusarium culmorum* indicated production of peptidases with optimum activity at alkaline pH range (Pekkarinen et al., 2002).

Pekkarinen et al. (2002) observed that the pH range from 8.3 to 9.6 showed highest proteolytic activity for *F. culmorum* and that in the stability test all activity was lost at pH 8.5. Possibly, in the pH with better activity, the enzymes are in conformation to react, with the active site exposed, becoming more susceptible to proteolytic action of other enzymes and consequently becoming less stable. The author points out that adding other proteins to the formulation apparently protect peptidase autolysis or inhibit conformational changes that could decrease stability. In comparison to others studies, Pekkarinen et al. (2002) showed that peptidase produced by *F. culmorum* became instable when incubated at temperatures over 45°C. Maia et al. (1999) showed greater stability at temperatures from 25 to 30°C for lipases produced by *Fusarium solani*, with inactivation after 60 min at 40°C for.

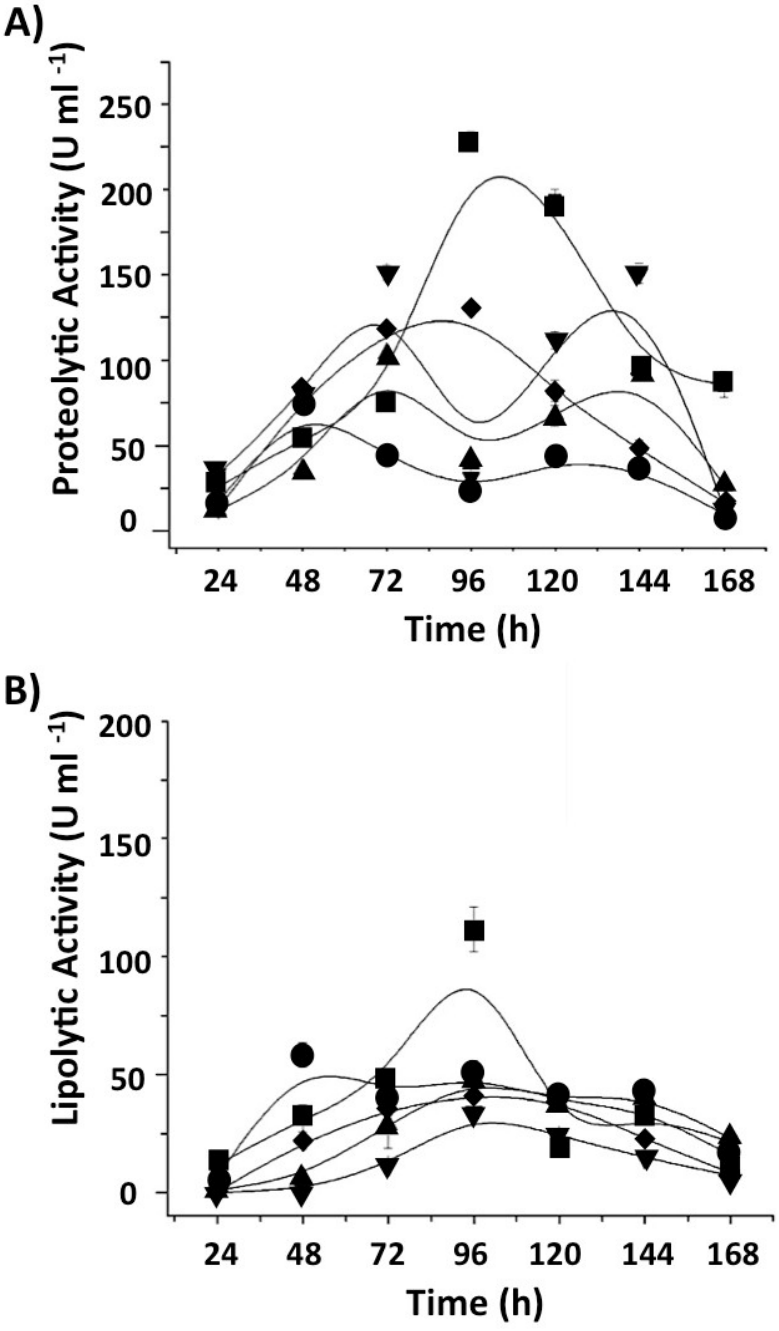

Figure 1. Proteolytic (A) and lipolytic (B) activity after 168 h of growth of *Fusarium oxysporum* in wheat bran and cottonseed meal in different proportions: 1:0 (■), 0:1 (●), 1:1 (▲), 3:1 (▼) and 1:3 (♦) (*P<0.05*)

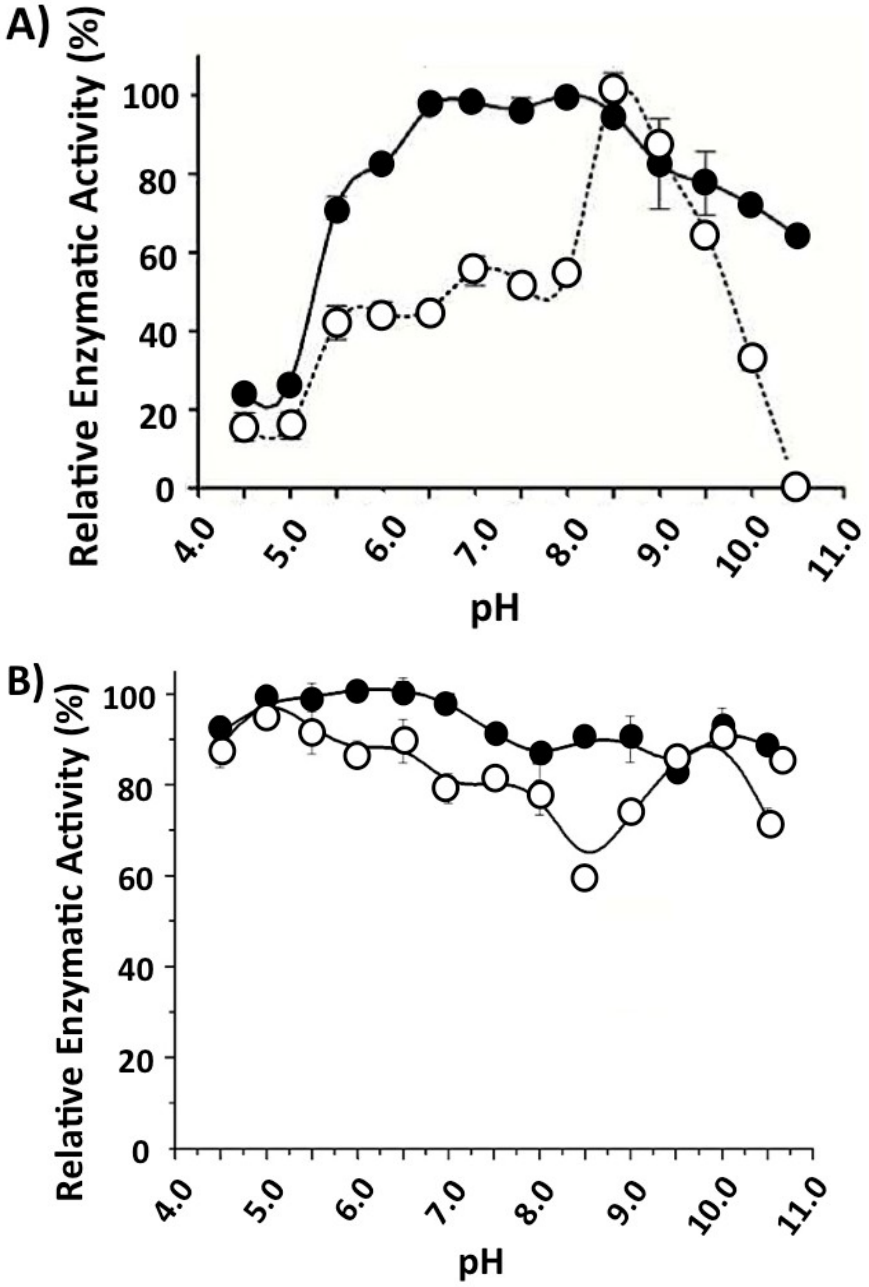

Figure 2. Effect of pH on the activity (A) and stability (B) of peptidases (●) and lipases (○) of the enzymatic extract produced by the fungus *Fusarium oxysporum* in solid-state fermentation bioprocess ($P<0.05$)

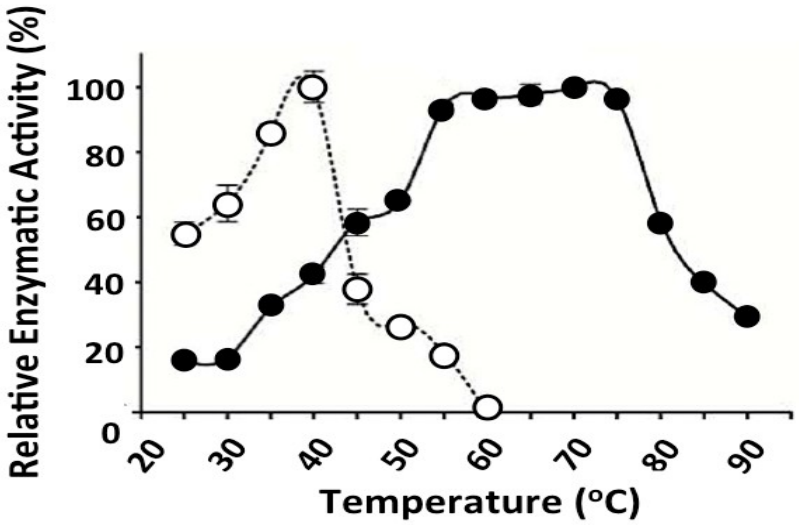

Figure.3 Effect of temperature on the activity of peptidases (●) and lipases (○) of the enzymatic extract produced by the fungus *Fusarium oxysporum* in solid-state fermentation bioprocess (*P<0.05*)

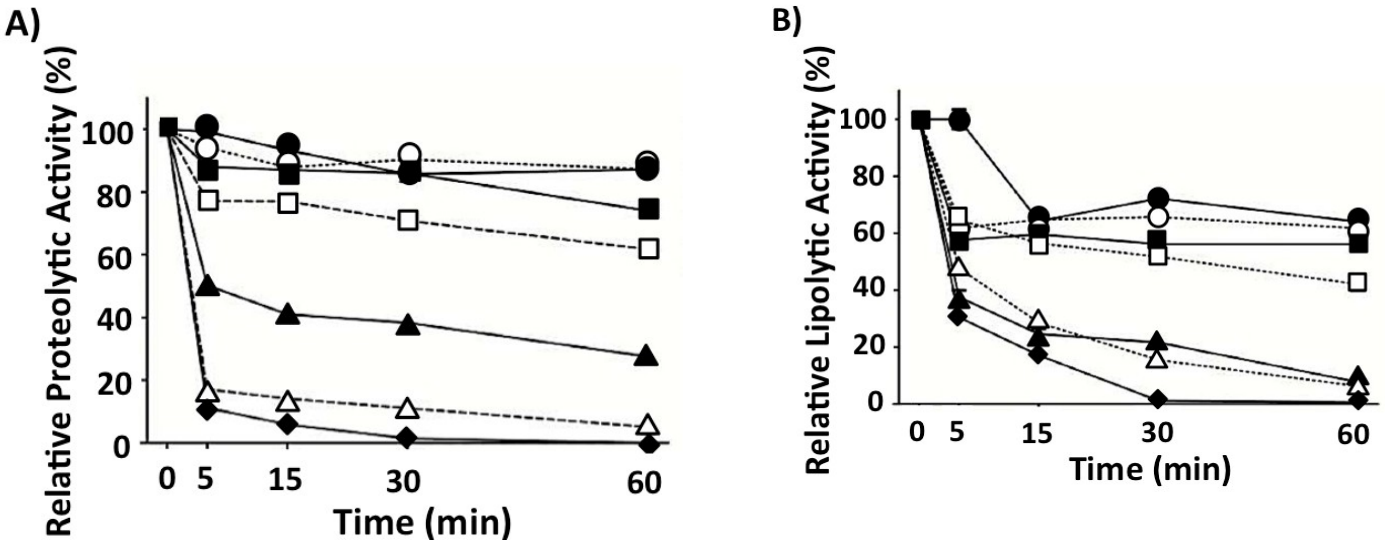

Figure.4 Effect of temperature on the stability of peptidases (A) and lipases (B) of the enzymatic extract produced by the fungus *Fusarium oxysporum* in solid-state fermentation bioprocess. Tested temperatures: 30 (●), 35 (○), 40 (■), 45 (□), 50 (▲), 55 (□) and 60°C (♦) (*P<0.05*)

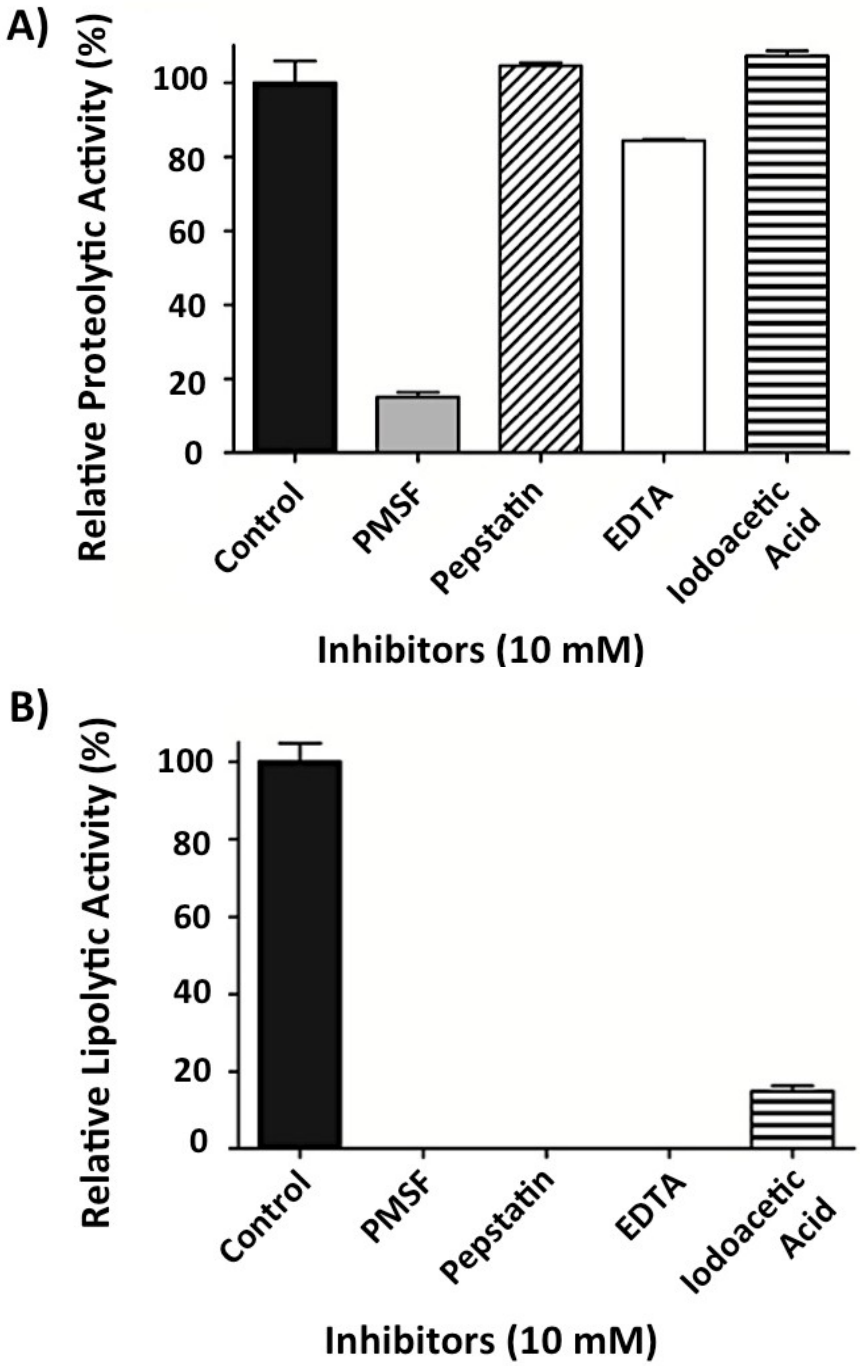

Figure.5 Effect of inhibitors on activities of peptidases (A) and lipases (B) of the enzymatic extract produced by the fungus *Fusarium oxysporum* in solid-state fermentation bioprocess ($P<0.05$).

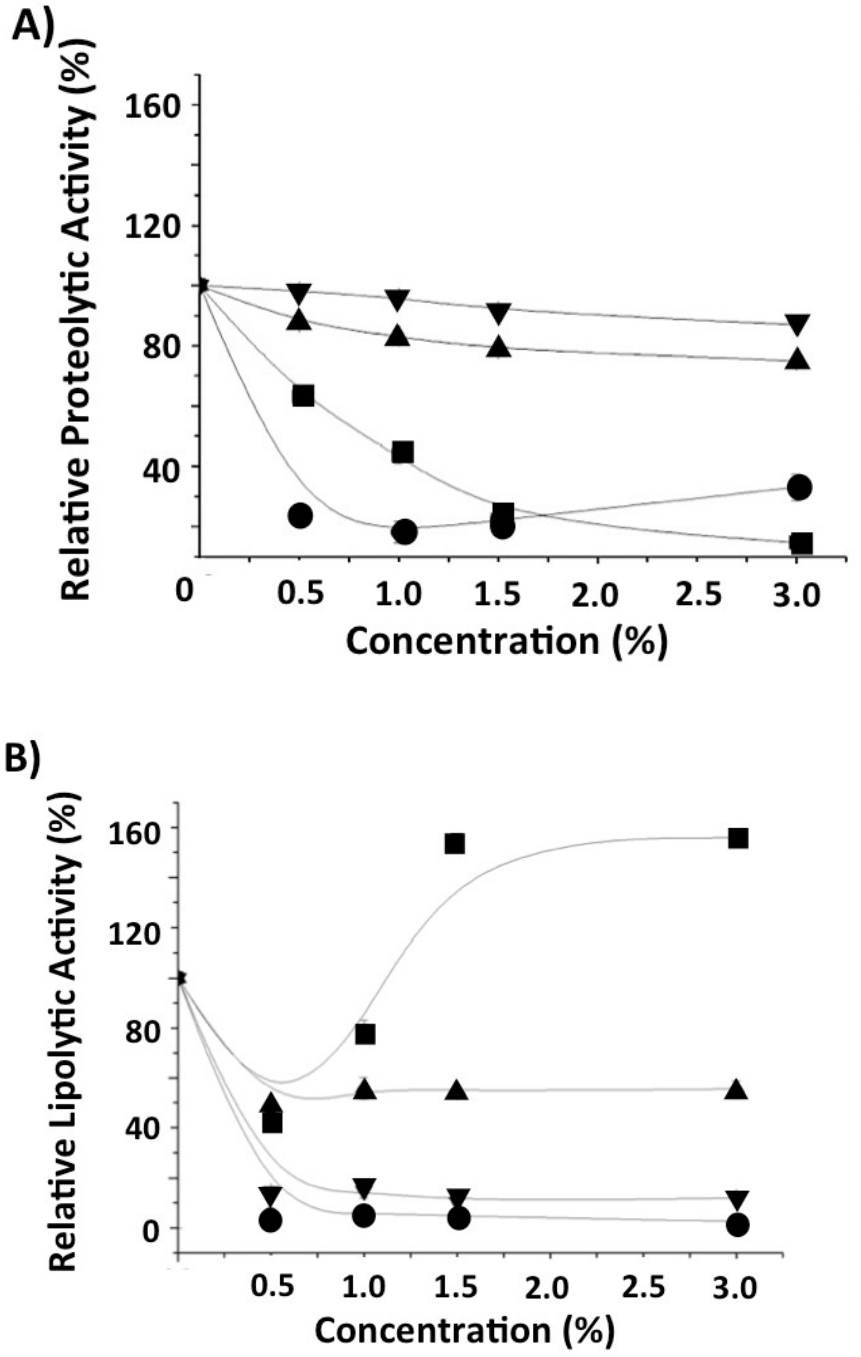

Figure.6 Effect of surfactants on activities of peptidases (A) and lipases (B) of the enzymatic extract produced by the fungus *Fusarium oxysporum* in solid-state fermentation bioprocess. Tested surfactants: CTAB (■), SDS (●), TRITON^{TM}X 100 (▲) and TWEEN$^{®}$80 (▼) (*P<0.05*)

The analysis of these parameters is important for planning the exposure time and temperature during the manufacturing processes and storage of the product. To ensure a considerable increase of the stability of the enzymes it is suggested to remove the surrounding water by techniques that enable the formulations to be in solid rather than liquid state (Namaldi et al., 2006).

In the effect of inhibition the peptidases produced in this work is in agreement with Pekkarinen et al. (2002) for peptidases from *F. culmorum*. Serine peptidases are the most important group of industrial enzymes, since it represents about 35% of sales of microbial enzymes. Its main application is in the detergent industry (Namaldi et al., 2006).

Effect of surfactants showed by Pahoja and Sethar (2002) describes experiments of different authors in which lipases were inhibited by Triton™100-X, Tween®80 and SDS. Studies (Alfani et al., 2000) indicate high activity of α-chymotrypsin when exposed to an environment rich in CTAB. According to the author, this behavior of activity promotion can be explained either by the phenomenon of reverse micelles or by a conformational change in the enzyme, resulting in greater affinity for the substrate.

The enzymatic activity of alkaline lipases and peptidases produced by the fungus *F. oxysporum* indicate considerable potential for application in the detergent industry. However, the effect of surfactants tests indicates that the choice of surfactant for the formulation should be in accordance with the purpose of higher lipolytic or proteolytic activity of the product. Another alternative is to formulate sophisticated enzymatic detergents without synthetic surfactants. These detergents find application in dry cleaning, dishwashing products, degreasers, pipe cleaning and wastewater treatment, among others (Hasan et al., 2006).

In addition to the detergent industry, other possible applications for enzymes are alkaline leather industry, paper industry, agrochemical synthesis of biopolymers and biodiesel (Hasan et al., 2006).

The use of agro-industrial waste as culture media for solid-state fermentation by fungi is an available and interesting way to obtain high added value products. The results allow us to say that the desired enzyme production can be achieved by the correct selection of the culture medium.

The produced enzymes in this study have optimum characteristics for a broad range of industrial and commercial applications. With appropriate adaptations, the obtained results can be extrapolated to other experiments with different microorganisms and enzymes.

Acknowledgments

We thank FAPESP (2011/06986-0), CAPES and CNPq for financial support.

References

Alfani, F., Cantarella, M., Spreti, N., Germani, R., and Savelli, G. 2000. α-chymotrypsin superactivity in cetyltrialkylammonium bromide-rich media. Appl. Biochem. Biotechnol. 88: 1-15.

Bhargav, S., Panda, B.P., Ali, M., and Javed, S. 2008. Solid-state fermentation: an overview. Chem.

Biochem. Eng. Q. 22: 49-70.

Demain, A.L. 2007. The business of biotechnology. Ind. Biotechnol. 3: 269-283.

Ducros, E., Ferrari, M., Pellegrino, M., Raspanti, C., and Bogni, C. 2009. Effect of aeration and agitation on the protease production by *Staphylococcus aureus* mutant RC128 in a stirred tank bioreactor. Bioprocess Biosyst. Eng. 32: 143-148.

Dunn, B.M. 2001. Determination of Protease Mechanism. In: Proteolytic enzymes: a practical approach (Eds) Beynon, R.J., and Bond, J.S. Oxford University Press, New York.

Gulati, R., Isar, J., Kumar, V., Prasad, A.K., Parmar, V.S., and Saxena, R.K. 2005. Production of a novel alkaline lipase by *Fusarium globulosum* using neem oil, and its applications. Pure Appl. Chem. 77: 251-262.

Gupta, R., Beg, Q.K., Khan, S., and Chauhan, B. 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl. Microbiol. Biotechnol. 60: 381-395.

Hasan, F., Shah, A., and Hameed, A. 2006. Industrial applications of microbial lipases. Enzyme Microb. Tech. 39: 235-251.

Hoshino, T., Sasaki, T., Watanabe, Y., Nagasawa, T., and Yamane, T. 1992. Purification and some characteristics of extracellular lipase from *Fusarium oxysporum* f. sp. *lini***.** Biosci. Biotech. Biochem. 56: 660-664.

Javed, M.M., Zahoor, S., Shafaart, S., Mehmooda, I., Gul, A., Rasheed, H., Bukhari, A.I., Aftab, M.N., and Haq, I-U. 2012. Wheat bran as a brown gold: Nutritious value and its biotechnological applications. Afr. J. Microbiol. Res. 6: 724-733.

Kanwar, S.S., Kaushal, R.K., Jawed, A., Gupta, R., and Swapandeep, S.C. 2005. Methods for inhibition of residual lipase activity in colorimetric assay: A comparative study. Indian. J. Biochem. Biophys. 42: 233-237.

Lotong, N., and Suwanarit, P. 1983. Production of soy sauce koji mold spore inoculum in plastic bags. Appli. Environ. Microbiol. 46: 1224-1226.

Maia, M.M.D., Morais, M.M.C., Morais, M.A., Melo, E.H.M., and Lima Filho, J.L. 1999. Production of extracellular lipase by the phytopathogenic fungus *Fusarium solani* FS1. Rev. Microbiol. 30: 304-309.

Merheb-Dini, C., Cabral, C., Leite, R.S.R., Zanphorlin, L.M., Okamoto, D.N., Rodriguez, G.O.B., Juliano, L., Arantes, E.C., Gomes, E., and da Silva, R. 2009. Biochemical and functional characterization of a metalloprotease from the thermophilic fungus *Thermoascus aurantiacus*. J. Agr. Food Chem. 57: 9210-9217.

Mitchell, D.A., Krieger, N., and Berovic, M. 2006. Solid-state fermentation bioreactors: fundamentals of design and operation. In: Verlag Nerlin Heidelberg (Eds). Springer, Germany.

Namaldi, A., Calik, P., and Uludag, Y. 2006. Effect of spray drying temperature and additives on the stability of serine alkaline protease powders. Dry Technol. 24: 1495-1500.

Nelson, P.E., Dignani, M.C., and Anaissie,

E.J. 1994. Taxonomy, biology, and clinical aspects of Fusarium species. Clin. Microbiol. Ver. 7: 479-504.

Pahoja, V.M., and Sethar, M.A. 2002. A review of enzymatic properties of lipase in plants, animals and microorganisms**.** Pakistan J. App. Sci. 2: 474-484.

Pekkarinen, A.I., Jones, B.L., and Niku-Paavola, M.L. 2002. Purification and properties of an alkaline proteinase of *Fusarium culmorum*. Eur. J. Biochem. 269: 798-807.

Rajmalwar, S., and Dabholkar, P.S. 2009. Production of protease by *Aspergillus* sp. using solid-state fermentation. Afr. J. Biotechnol. 8: 4197-4198.

Rao, M.B., Tanksale, A.M., Ghatge, M.S., and Deshpande, V.V. 1998. Molecular and biotechnological aspects of microbial proteases. Am. Soc. Microbiol. 62: 597-635.

Rifaat, H.M., El-Mahalawy, A.H., El-Menofy, H.A., and Donia, S.A. 2010. Production, optimization and partial purification of lipase from *Fusarium oxysporum*. J. App. Sci. Environ. Sanit. 5: 39-53.

Sarath, G., Zeece, M.G., and Penheiter, A.R. 2001. Protease assay methods. In: Proteolytic enzymes: a practical approach (Eds) Beynon, R.J., and Bond, J.S. Oxford University Press. New York.

Shu, Z.Y., Jiang, H., Lin, R.F., Jiang, Y.M., Lin, L., and Huang, J.Z. 2010. Technical methods to improve yield, activity and stability in the development of microbial lipases. J. Mol. Catal. B: Enzyme. 62: 1-8.

Silva, R.R., Ângelo, T., and Cabral, H. 2013. Comparative Evaluation of Peptidases Produced by *Penicillium corylophilum* and *Penicillium waksmanii* in Solid State Fermentation Using Agro-industrial Residues. J. Agr. Sci. Tech. B. 3: 230-237.

Treichel, H., Oliveira, D., Mazutti, M.A., Di Luccio, M., and Oliveira, J.V. 2010. A review on microbial lipases production. Food Bioprocess. Tech. 3: 182-196.