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RESEARCH ARTICLE

Utilization of tomato pomace as a substrate for neutral protease production by *Aspergillus oryzae* 2220 on solid-state fermentation

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Abstract

Neutral protease production under solid-state fermentation was investigated by *Aspergillus oryzae* NRRL 2220, using tomato pomace as substrate and enzyme inducer. This substrate is composed of more than 52% of insoluble fiber, more than 20% of protein, 6.52% of ash and other organic materials. This is used as support and nutrients for the microorganism. Initial cultures were performed in conical flasks. Best protease production was obtained after 72 hrs, with 50% initial moisture content, pH of 6 and 1 ml (2 x 10^7 spores) of 5 days inoculum. Enrichment with soy bean flour induces an increasing of 66.5% of protease activity. The production of protease in pilot bioreactor yielded 12 U/gds after 42hrs of culture. It was enhanced by 13% with a superior result of protease activity (13.55 U/gds) using tomato pomace without enrichment. The present work indicates the potentiality of this waste for fermentation use especially for fungal protease production.

Introduction

Proteases are the most important industrial enzymes, accounting for nearly 65% of the global enzyme market. They have numerous applications in various industries: food, pharmaceutical, detergent, leather dehairing, silver recovery from used X-ray films, cosmetic and peptide synthesis (Rodríguez Couto, 2008; Vishwanatha et al., 2010). Microbial proteases account for forty percent of the total worldwide enzyme sales (Lazim et al. 2009); they have been produced either by submerged or solid-state fermentation (SSF). This last one has been established as a superior technique for the production of enzymes (Sandhya et al., 2005; Belmessikh et al., 2013); its advantages include simplicity, lower production costs, low wastewater output and high enzyme yields. SSF is similar to the natural habitat of moulds, which are the best-adapted species for this process. This can be attributed to the advantage of the hyphal mode of growth with the utilization of variable solid substrates (Ustok et al., 2007). There is a great influence of the nature of solid substrate in SSF system for the production of microbial enzymes. Although a number of substrates have been employed for cultivating different microorganisms, wheat bran has been the preferred choice for microbial protease production compared to many other agro-industrial residues (Krishna, 2005; Mukherjee et al., 2008; Sandhya et al., 2005).

Thus, it is desirable that raw material intended for fermentation purposes contain as high content of protein as possible. Some proteases are produced and secreted only in the presence of proteins in the medium (Abidi et al., 2008). In this respect, the residue from tomato processing is interesting substrate. Tomato pomace consists of peels, seeds and some pulp of the fruit, contains protein levels similar to those of soybean and sunflower proteins (Tsatsaronis and Boskou, 1975; Assi and King, 2007). It has been used in submerged fermentation for the production of enzymes from bacterial strains (Rawashdeh et al., 2005) and fungal strains (Do Rosario Freixo et al., 2008). Only a few studies have focused on the use of these wastes as a substrate in SSF (Carvalheiro et al., 1994; Assi and King, 2007), to our knowledge, the literature shows no study on its utilization for the production of proteases. In our previous studies, tomato pomace was used in submerged fermentation for protease production by *Aspergillus oryzae* (Ahlburg) Cohen 1042.72 (Boukhalfa-Lezzar, 2010); and later, in a comparative study between solid and submerged fermentations for neutral protease production by *A. oryzae* 2220 (Belmessikh et al., 2013). This paper presents the study of tomato pomace composition, optimization of some parameters of SSF for neutral protease production by *A. oryzae* and its evaluation in pilot fermenter for the scale up production.

1. Material and methods

1.1. Microorganism and inoculum preparation

The strain *Aspergillus oryzae* NRRL 2220 was maintained on potato dextrose agar at 4 °C after growing at 30 °C for seven days. The spores from a fully sporulated Petri dish were dispersed in 10 ml of 0.1% Tween 80 by a sterile pipette under aseptic conditions. The suspension obtained was used as inoculum, which contains $2x10^7$ spores /ml.

1.2. Substrate

Tomato pomace was obtained from a tomato paste manufacturing unit (« Maison LATINA, Groupe OUCHERIF des industries alimentaires », Chelghoum-Laid, Algeria). It was drained, dried and placed in bags at room temperature until use. The analysis of this by-product was accomplished by INZO Laboratory (Château-Thierry, France).

1.3. Solid- state fermentation in flasks

Twenty grams of tomato waste were weighed into 500 ml Erlen-meyer flask. Distilled water or buffer solution, was added to the desired moisture level (see below). The flasks were autoclaved at 121 °C for 20 min and inoculated after cooling. The contents were mixed and incubated at 30 °C for desired time.

1.4. Effect of process parameters on protease production

Some process variables were studied to monitor their effect on neutral protease production in SSF. These were : fermentation time (0, 24, 48, 72, 96, 120 and 144 h), initial moisture content of the substrate (40, 45, 50, 55, 60, 65 and 70%), initial pH (3, 4, 5, 6, 7 and 8); inoculum size (0.1, 0.25, 0.5, 1 and 2 ml), inoculum age (3, 4, 5 and 6 days) and additional soybean flour (SBF) as nitrogen source (at 5, 15, 25 and 35 mg/g of substrate). Process variables were varied individually in separate experiments. The optimum value determined for one parameter was applied in subsequent experiments. Initial SSF of tomato pomace was carried out at 50% of moisture content and 1 ml of one week old inoculum.

1.5. Solid-State Fermentation in bioreactor

Pilot scale production of neutral protease was carried out in a pilot fermenter (Fujiwara, Japan). Through the substrate humidified air is continuously forced from the bottom. For this study, two types of fermentation medium were tested: tomato pomace supplemented with SBF (15 mg/g of substrate) and tomato pomace without any supplementation. The quantity of 5 kg of substrate were taken in poly propylene bags (2.5 kg/ bag) and autoclaved at 121°C for 20 min. The cooled medium was inoculated by 5 Erlen-meyers flasks (1 flask/kg) and thoroughly mixed. Each inoculum flask contains 20 g of the substrate inoculated with 1 ml of spore suspension and incubated for 5 days at 30 °C. The inoculated substrate was loaded on bioreactor perforated tank, where the thickness of the substrate bed was 10 cm. The moisture content of the medium after inoculation was 50%. The fermenter was carried out for 44 hours at 30 ± 4 °C. Periodically, it was necessary to spray sterile distilled water and agitate the medium during the cultivation.

1.6. Extraction of the enzyme

After incubation, the fermented mass was mixed with distilled water (1:5, w/v) in a Waring-blender for 40 second. The suspension was filtered under vacuum and the filtrate was centrifuged (Jouan MR 1812) at 10,000 x g for 10 min (4 °C). The supernatant was used as crude enzyme extract.

1.7. Protease assay

Protease activity was assayed by Anson method (1938) as modified by Mechakra et al. (1999) using casein as substrate and with sodium phosphate-citrate as buffer (pH 6.8). One unit of protease activity was defined as the amount of enzyme that liberated 1 µmol tyrosine per minute under assay conditions and reported in terms of protease activity per gram of initial dry substrate (U/gds). All experiments were conducted in three sets and the data presented here are the mean of triplicate determinations ± S.D.

2. Results and discussion

2.1. Substrate chemical composition

The composition of dried tomato pomace is shown in Table 1. This substrate is composed of 52% of insoluble fiber (lignin and cellulose). This composition maintains the substrate structure even after sterilization, which make it suitable for SSF as support and a nutrient source (Krishna, 2005). Protein content of 20.1% is similar to those obtained by Tsatsaronis and Boskou (1975) and Kramer and Kwee (1977). However, they are higher than results reported for wheat bran by Naivikul in 1997 (12-17%) and for rice bran by Fabian and Ju in 2011 (about 10-15%). The presence of minerals (6.52%) is similar to the results reported by Tsatsaronis and Boskou (1975). Thus, the use of tomato pomace as a substrate for SSF seems appropriate and deserves to be explored.

2.2. Neutral protease production profile

The time course of protease production was examined. During the first day of incubation, the mould grew very fast with the formation of a white mat mycelial on the solid substrate. Thereafter, a green-yellow mass began to appear, indicating that sporulation had occurred. Fig. 1 shows the proteolytic activities produced by *A.oryzae*, under SSF using tomato pomace. Enzyme production started after the first day of culture, maximum production (6.21 U/gds) was reached at 72 h. The subsequent decrease in enzyme activity with increasing fermentation time could be due to cessation of production, or to enzyme deactivation (Sumantha et al., 2005).

2.3. Effect of initial moisture content

Initial moisture content of the substrate has a great influence on growth and the physicochemical properties of solids, which, in turn, affects productivities in SSF. For filamentous fungi, the moisture levels could vary between 20-70% (Krishna, 2005). During the fermentation of *A.oryzae* 2220, the effect of initial moisture on protease production is shown in Fig.2 (A). Protease activity remained largely unchanged. The enzyme production was similar at any given moisture from (40 to 70%). Solid-state fermentation of wheat bran by *A. oryzae* NRRL 1808 for protease production showed optimum yield at 43.6% of moisture (Sandhya et al., 2005), however *A. oryzae* NCIM 649 exhibited maximum protease activity at 60% moisture initially added in the bran (Agrawal et al., 2005) for *Rhizopus oryzae*, it was of 140% (Tunga et al., 1998). It is clear that for the same substrate, optimal moisture content for protease production differs for each fungal strain. As the optimal value of moisture content depends on both the microorganism and the solid matrix used. So, 50% of initial moisture content was selected for further optimization of the extracellular protease production.

2.4. Effect of initial pH

In order to study the effect of medium pH on protease production, experiments were performed with sodium phosphate-citrate or sodium phosphate buffer at different initial pH before sterilization of substrate and incubated for 72 h. The effect of varying initial pH values on enzyme production is shown in Fig. 2 (B). Optimum production was obtained at pH 6 (7.35 U/gds). This is in accordance with the results obtained by Tunga et al. (1998). However Sandhya et al., (2005) reported no significant differences in the yield of neutral protease production by *A.oryzae* NRRL 1808 on wheat bran SSF. Agro-industrial residues possess excellent buffering capacity especially when they are used in SSF (Shankar and Mulimani, 2007). Final pH values observed at the end of fermentations show an increase of pH; this could be related to ammonia production in the culture medium as a result of protein metabolism after protease action.

2.5. Effect of inoculum size

Size of inoculum is an important biological factor for growth and metabolite production. There was a gradual increase in the enzyme synthesis with increase in inoculum volume up to 1 ml containing 2 x 10^7 spores (7.7 U/gds), but thereafter, a decline was observed (Fig.3 (A)). Higher inoculum size did not increase enzyme production; this might be due to increased competition for carbon source and nutrients. Hence, a balance between the proliferating biomass and available material will yield maximum enzyme production. This is in accordance with the results of many authors (Mukherjee et al., 2008; Sandhya et al., 2005; Tunga et al., 1998).

2.6. Effect of inoculum age

Maximum neutral protease was obtained with an inoculum age of 5 days (Fig.3 (B)), where the enzyme activity reached a value of 6.57 U/gds. The 6 days inoculum revealed an activity decrease about 19%. Aikat and Bhattachayya (2000) have reported that protease production by *Rhizopus oryzae* required an inoculum age of 7 days. Control of spore age at inoculation of fermentation is important for growth and metabolite production. The inoculum development time may generate premature or old and non-viable spores.

2.7. Effect of supplementation with nitrogen source

Although tomato pomace contains good amount of protein, supplementing it with SBF leads to interesting observations. An increase in protease activity was observed at all the levels tested (Fig. 4). More than 66.5 % was reached with 15 mg of SBF/g of substrate. It was reported that casein and soy protein increased protease production by *A. niger* var. tieghem (Chakraborty et al., 1995), by *A.oryzae* NCIM 649 (Agrawal et al., 2005) and by *A.oryzae* MTCC 5341 (Vishwanatha et al., 2010). Prakasham et al. (2006) revealed that complex nitogen sources supported better protease production over inorganic nitrogen compound which supports the present findings.

2.8. Protease production in Bioreactor

The cultures in the pilot fermenter, a Koji bioreactor, showed that protease production began 18 h after inoculation; the maximum activities were reached after 42 h of incubation (Fig. 5). At this stage, 12.09 U/gds of protease was obtained when tomato pomace was supplemented with SBF; and 13.55 U/gds, highest yield, without supplementation. From these results, it is clear that the protein content of tomato pomace is sufficient to induce protease production. Aeration in Koji bioreactor has significant effect on enzyme productivity. According to Tunga et al. (1999), protease production through aeration is obviously more than without aeration system. The forced aeration of humid air allows alleviating oxygen gradients without lowering the moisture content of the bed (Raghavarao et al., 2003). This helps microbial growth, heat removal from the substrate and results in better protease production. Many workers (Anisha et al., 2010; Pandey et al., 2008) have reported enhancement of enzyme yields by oxygen enrichment.

Table 1. Chemical composition of dried tomato pomace.

Component	**%(w/w dry basis)**
Ash	6.52
Moisture	6.97
Protein	20.1
Fat	11.51
Starch (Ewers)	2.5
Total insoluble fibers	52.4
Lignin	24
Cellulose	19.5
Hemicellulose	8.9

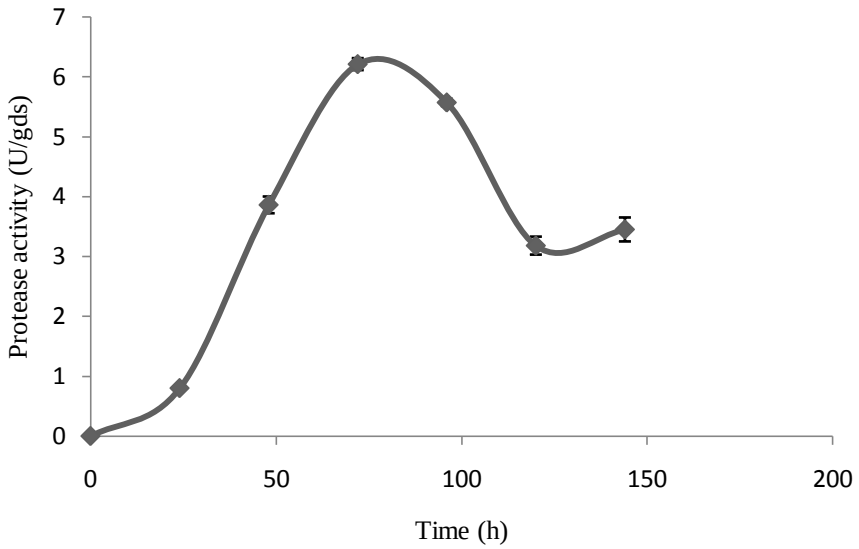
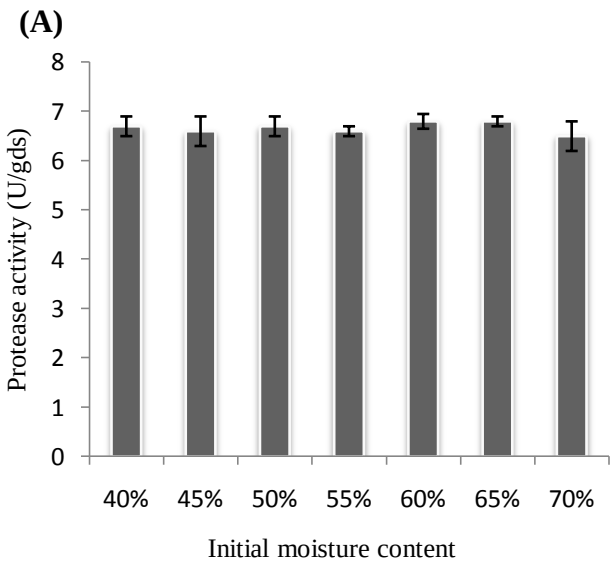


Figure 1. Progress of neutral protease activity during tomato pomace SSF by A.oryzae 2220.



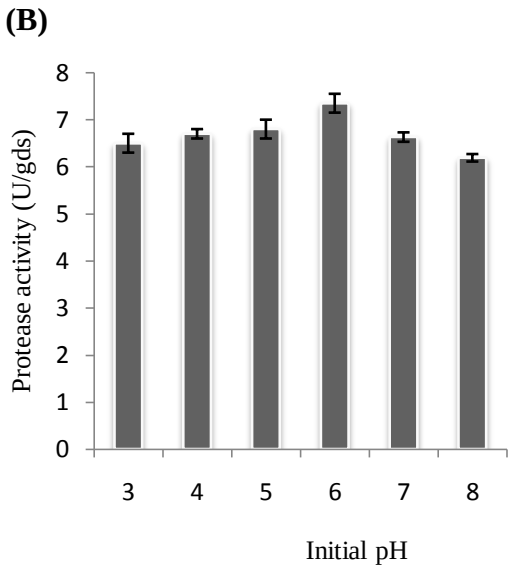
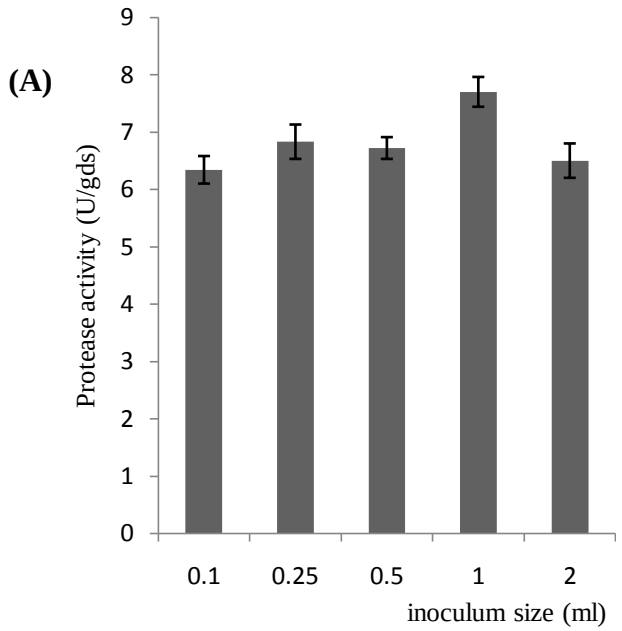


Figure 2. Neutral protease production by A.oryzae 2220 in SSF at (A) different initial moisture content and (B) different initial pH of the fermentation medium.



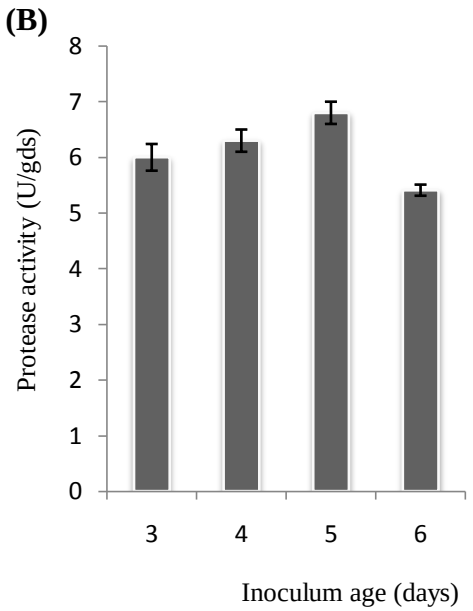


Figure 3. Neutral protease production by A.oryzae 2220 in SSF at (A) different inoculum size and (B) different spore age.

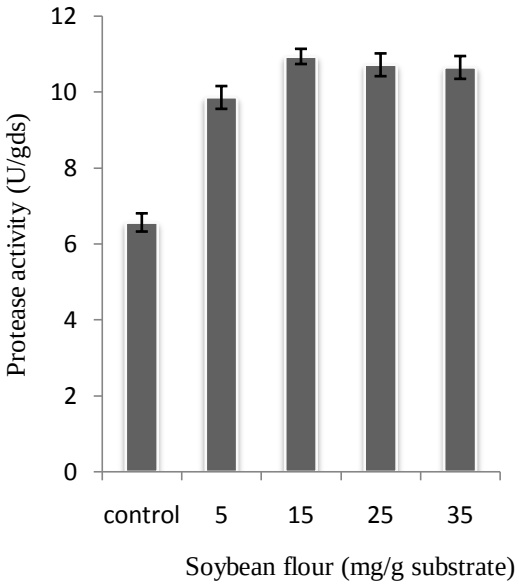


Figure 4. Effect of SBF concentration on neutral protease production. Fermentation medium without supplementation was taken as control.

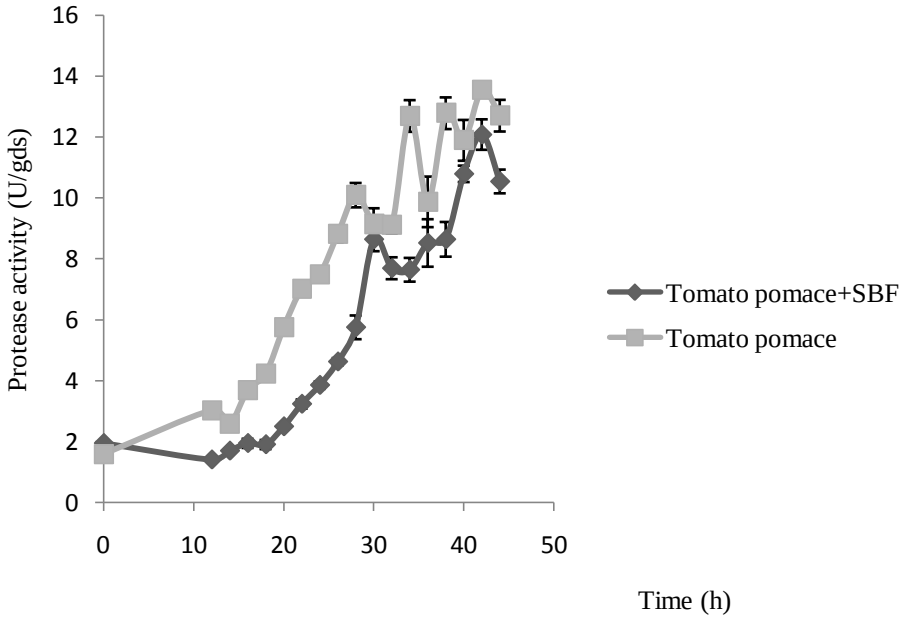


Figure 5. Profile of neutral protease production by A.oryzae 2220 when grown in bioreactor, on tomato pomace and tomato pomace with SBF.

3. Conclusion

In this study we suggested an approach for the valorization of tomato pomace under solid-state fermentation to produce neutral protease. Tomato pomace used as sole substrate in optimized conditions afforded good levels of protease (13.55 U/gds). The process is simple and environmentally-friendly system without pretreatments. It could be scaled-up for large production. The protease produced by *A.oryzae* on tomato pomace, is free of undesirable flavor that is advantageous for its use in food and pharmaceutical industries.

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