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OPTIMIZATION OF MILK-CLOTTING PROTEASE PRODUCTION BY A LOCAL ISOLATE OF *ASPERGILLUS NIGER* FFB1 IN SOLID-STATE FERMENTATION

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ABSTRACT

The need to surmount the limitation of obtaining rennin, has been actively pushed researches to find new substitutes that present high milk-clotting activity which enables the production of high yields of cheese. In this study, the production of extracellular milk-clotting protease by locally isolated fungal specie, *Aspergillus niger* FFB1 under solid-state fermentation (SSF) using cheep agro-industrial byproduct (wheat bran) was optimized. The effects of several physicochemical and environmental factors were investigated to select the optimal conditions that ensure the best milk-clotting activity by application of "One-factor-at-a-time" method. A trial of cheese production using the crude extract was also carried out. The maximum enzyme activity (830 SU/g bran with a ratio MCA/PA of 4.25) was obtained under the optimum conditions of temperature (30°C), spores concentration (10^6 spores/mL), incubation time (72 hours), and moisture content of solid substrate (39.2%) adjusted suitably with mineral solution (Czapek-Dox) of pH 4.

Keywords: *Aspergillus niger*, milk-clotting enzyme, protease, optimization, solid-state fermentation

INTRODUCTION

Cheese-making and fermentation represent the first examples of applied biochemistry and biology (**Fox, 1999**). The essential characteristic step in the manufacture of all cheese varieties involves coagulation of the casein, the main component of the milk protein system, to form a coagulum. The majority of cheeses are produced by enzymatic coagulation. Calf chymosin (an aspartic protease) from the stomachs of young calves was used traditionally as the main milk coagulant for cheese production.
The caseins exist as micelles stabilized by a surface layer of κ-casein. In the presence of chymosin, milk-clotting occurs in two separate steps: the first phase starts with a specifically cleavage of κ-casein at the Phe_{105}-Met_{106} bond which results in the release of hydrophilic glycopeptide (caseinomacropeptide) that passes into the whey and *para*-κ-casein remained attached to the casein micelle. The non enzymatic secondary phase starts when 85-90% of the total κ-casein has been hydrolyzed; the aggregation of *para*-κ-casein and other caseins (αs_1, αs_2, β and γ) occurs under the influence of Ca^{2+} and eventually results in gel formation (**Law, 1997, Fox *et al.*, 2004**).
The limited supplies of such rennets due to the increasing trend to slaughter calves at an older age than previously, together with a worldwide increased demand for cheese, has led to a shortage of calf rennet. Consequently rennet substitutes from different origins are now used widely for cheese manufacture with more or less satisfactory results (**Kumar *et al.*, 2005**; **El-Bendary *et al.*, 2007**; **Kumari *et al.*, 2012**).
Aspartic proteases from fungal origin are inactivated at normal pasteurization temperatures and have low levels of non-specific proteolytic activity. The majority of these enzymes are mainly produced by genera such as *Aspergillus, Mucor, Entothia, Rhizopus, Penicillium* and *Fusarium.* These fungi are characterized by their adaptation to solid-state fermentation (SSF) using cheap substrates, which improve the recovery of extracellular enzymes from fermentation media with high yields, solve pollution problems and reduce the capital costs of process (**Vishwanatha *et al.*, 2010**; **Sathya *et al.*, 2009**).
Solid-state fermentation involves the growth of microorganisms on solid substrates in the absence or near absence of free water in the space between particles. This low moisture content makes the SSF different from the submerged fermentation (SmF) (**Manpreet *et al.*, 2005**). Research on the selection of appropriate substrates for SSF was mainly focused on natural agricultural products, agro-industrial wastes and by-products, where the solid substrate not only ensures the supply of nutrients to the culture, but also serves as a support for the microbial cells (**Couto and Sanroman, 2006**).
Application of SSF conditions to upgrade the nutritive value of agricultural residues and to produce a wide variety of enzymes, mainly from mold origin, has been increased especially when higher yields can be obtained than in SmF process (**Graminha *et al.*, 2008**).
The aim of our study is to bring together different factors that affect the production of milk-clotting protease from *Aspergillus niger* FFB1 by solid-state fermentation and to deduce their optima. More consideration is given to topics related to cheese-making, where obtaining a low proteolytic activity compared to milk-curdling one must be respected.

MATERIAL AND METHODS

Microorganism and maintenance of culture

The fungus strain used in this work was isolated from soil samples of oak forest (Boumerdes region's) and identified as *Aspergillus niger* FFB1 by the fungus culture collection (MUCL) of the Louvain-la-Neuve University (Belgium) after cultural and morphological studies. In addition, this strain was found unable to produce Ochratoxin A after analysis by High Performance Liquid Chromatography (HPLC) of extracts obtained from *A. niger*' cultures on Czapek yeast agar (CYA) medium (**Fazouane-Naimi *et al.*, 2010**). The strain was maintained on potato dextrose agar slants at 4°C and subculture for each two months on the same medium to preserve its vitality.
The inoculum for fermentation process was prepared by scarping off the spores from cultures grown on potato dextrose agar (28±2°C for 5-7 days) in a sterile solution of 0.1% Tween-80 (10ml/ Petri plate) under aseptic conditions (**Agrawal *et al.*, 2005**; **Sandhya *et al.*, 2005**). After filtration through Whatman paper no.1, the absorbance of the suspension was measured at 650nm (**Markwell *et al.*, 1989**). The spore suspension was diluted to produce a final absorbance of 0.55 in a 1cm cuvette. This concentration is equivalent to $2x10^6$ spores/mL determined by counting in Malassez cell under optical microscope. 1mL of this suspension was used as inoculum.

Screening of protease production by plate agar

Aspergillus niger FFB1 was screened for protease production using modified Czapeck-Dox Agar medium containing : Saccharose (30 g/L), $NaNO_3$ (3 g/L), K_2HPO_4 (1 g/L), $MgSO_4.7H_2O$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4.7H_2O$ (0.01 g/L), Casein (1 g/L), Agar (15 g/L) with pH=6.2. The medium was inoculated with fungal hyphae discs and incubated at 28±2°C. After 5 days, the radial growth of colonies and substrate clearing zones were measured (**Agrawal *et al.*, 2005**).

Screening of protease production by SSF

Wheat bran was used as substrate to test quantitatively the proteolytic activity of *A. niger* FFB1. A quantity of 5g of substrate was taken in 250mL Erlenmeyer flasks moistened with 4mL of mineral solution containing : NH_4NO_3 (5 g/L), KH_2PO_4 (2 g/L), $MgSO_4.7H_2O$ (1 g/L) and NaCl (1 g/L). After sterilization (121°C for 20 min) and cooling, the mixture was inoculated with 1mL of spore suspension and incubated at 28±2°C for 4 days (**Sumantha *et al.*, 2005**).

Solid-state fermentation

The substrate used in this study, wheat bran, was obtained from a local market in Algeria, it was characterized to determinate the different components (Carbohydrates 62.5%, Lipids 6%, Total proteins 16.6%, Moisture content 6.4%). Wheat bran was milled to reduce the particles size (between 0.425 and 0.85mm) then stored in sterile bottles. Fermentations were carried out in 250mL Erlenmeyer flasks containing 10g of wheat bran mixed with mineral medium. The flasks were autoclaved at 121°C for 20 min, cooled, then inoculated with 1mL of spore suspension and incubated stationary. All fermentations and assays are conducted in triplicate and each result is the average of three trials ± SD (Standard Deviation).

Optimization of process parameters for milk-clotting protease production

The method adopted for the optimization of SSF parameters is the design "One factor at a time", based on the variation of one independent variable over a desired range while fixing all others variables at a certain level. This strategy is used to evaluate the individual effect of one factor (parameter) and then include its optimal value before proceeding to the next factor optimization (**Kumar *et al.*, 2003; Wang and Wan, 2009**).

In addition to ammonium sulfate solution (AS) at 0.05%, three moistening mineral solutions were tested for maximum protease production:

- Czapek-Dox solution: $NaNO_3$ (2.5 g/L); KH_2PO_4 (1 g/L); $MgSO_4.7H_2O$ (0.5 g/L); KCl (0.5 g/L)
- M-9 solution: NH_4NO_3 (3 g/L); KH_2PO_4 (1 g/L); $MgSO_4.7H_2O$ (1 g/L); $FeSO_4.5H_2O$ (0.01 g/L) **(Tunga *et al.*, 1998**).
- SM solution: NH_4NO_3 (5 g/L); KH_2PO_4 (2 g/L); $MgSO_4.7H_2O$ (1 g/L); NaCl (1 g/L) (**Sumantha *et al.*, 2005; Paranthman *et al.*, 2009**).

After the selection of the mineral solution, different culture conditions influencing milk-clotting protease production such as incubation temperature (20, 25, 30, 35, 40 and 45°C), initial pH of the medium (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0), total moisture content (before autoclaving) of wheat bran (39.2, 54.3, 62.5, 65 and 70.8%), spore concentration (ranging from 1 to $5x10^6$ spores/mL) and incubation time (0, 24, 48, 72, 96, 120, 144 and 168 hours) were optimized.

Extraction of crude enzyme

At the end of fermentation, the milk-clotting protease of *A. niger* FFB1 was extracted with distilled water. The moldy bran was taken up in 50mL of sterile distilled water corresponding to a ratio of 1:5 (w/v). After shaking on a rotary shaker (160 rpm/min, 2 hours, 30°C) (**Tunga *et al.*, 2003**), the homogenized mixture was centrifuged at 2504 g for 30 min at 4°C. The clear supernatant recovered represents the crude enzyme extract used for analytical studies.

Measurement of milk-clotting activity

Milk-clotting activity (MCA) was determined as described by **Arima *et al.*** (**1970**). 10g of skim-milk powder was dissolved in 100mL of a 10mM calcium chloride solution. After pH was adjusted to 6.4, 1mL (E) of the supernatant was added to 10mL (S) of the milk pre-incubated at 35°C for 15 min. The end point recorded was the time (T en seconds) it took for small clots to become visible to the naked eye. Milk-clotting activity is expressed in term of Soxhlet Unit (One unit was defined as the amount of enzyme that clotted 1mL of substrate within 40 min at 35°C) and calculated using the following equation: ***MCA (SU/mL) = 2400/T x S/E***

Estimation of total protein content

Quantitative estimation of protein in the crude extract was determined by the method of **Bradford** (**1976**) using Bovine Serum Albumin (BSA) for the standard curve. Concentrations were expressed as mg protein/g of dry fermented substrate.

Proteolytic activity (PA) determination

A modification of Anson's method (**Anson**, **1938**) was used to assess the proteolytic activity. A reaction mixture containing 2.5mL of 2.5% casein prepared in sodium-citrate buffer (0.1M, pH 5.2) and 0.5mL of the crude enzyme was incubated at 40°C for 30 min. The reaction was then stopped by adding 5mL of 4% (w/v) trichloroacetic acid chilled. The mixture was filtrated to remove the precipitate, then to 1ml of supernatant, 2.5mL of 2% Na_2CO_3 and 0.5mL of diluted Folin Ciocalteau's phenol reagent were added. After incubation for 30 min in obscurity, the absorbance of the mixture was read at 750nm using tyrosine as standard for curve preparation. One unit (U) of enzyme activity represents the amount of enzyme required to liberate 1µg of tyrosine from substrate (casein) per hour under assay conditions.

Cheese making with the crude extract of *A. niger*

Pressed and unripened cheese was manufactured from cow milk by using the milk-clotting protease produced by *A. niger* FFB1. Raw milk (28% of fat) containing calcium chloride (0.2 g/L) was pasteurized at 90°C for 2min. Pasteurized milk, divided into two equal portions, was cooled to 38-40°C and maintained at this temperature in a water bath. Milk was then coagulated by adding commercial rennet (0.03 g/L) (pure chymosin: 1300IMCU/g, CHY-MAX, CHRHANSEN, Denmark) for the first portion, while for the second one, 15ml of the crude extract produced by *A. niger* was added. A firm coagulum was formed after 40min; the whey was then removed and replaced with hot water. After drainage (4 hours at 28°C), the curds were scooped into cylindrical moulds and pressed for 1hour. The cheeses were brine salted (2% NaCI) for 24 hours at 4°C. Samples were taken for physicochemical and sensory analyses.
Fat content of cheese was measured by Van-Gulik's method. The moisture content and the dry extract content were determined by oven drying the cheese samples (2.5 g) at 85°C to constant weight. pH was determined by placing directly the electrode of the pH meter into cheeses (**Amariglio, 1986**).

RESULTS AND DISCUSSION

Screening of protease production

Production of extracellular enzymes is determined by incorporation of test substrate into a basic medium. The agar containing casein revealed that *A. niger* FFB1 exhibits a protelolytic activity indicated by the clear zone around the colony created after hydrolysis of casein to soluble peptides and amino-acids (Fig. 1). The hydrolysis zone of 28mm produced could be related to the amount of protease released by the fungus in the medium. Wheat bran in SSF allowed to evaluate the milk-clotting activity (35.7 ± 3.8 SU/mL) and the proteolytic one (1253.01 ± 145.07 µg/h/mL) of proteases produced by *A. niger* FFB1.

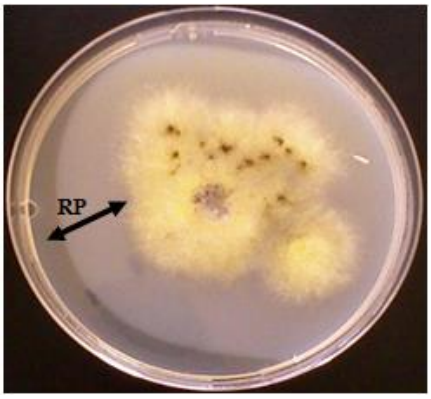

Figure 1 Screening of proteolytic activity of *A. niger* FFB1 on modified Czapek-Dox agar medium (RP: Radius of Proteolysis; culture of 5 days at 28°C).

Factors affecting milk-clotting protease production

Choice of mineral medium

As shown in Figure 2, the addition of inorganic salts has a distinct effect on enzyme production. The results demonstrate that Czapeck-Dox solution added to wheat bran ensures increasing in production of enzyme with milk-clotting activity 519.71±49.473 SU/g bran probably related to the presence of KCl in the medium.
Maximum alkaline protease activities of *Rhizopus oryzae* NRRL 21498, *Bauveria felina* and *Aspergillus oryzae* NCIM 649 have been reported using M-9 solution

as moistening medium of wheat bran by **Tunga *et al.* (1998)** and by **Agrawal *et al.* (2005)** respectively. **Wu *et al*. (2008)** reported that addition of metallic salts to basal medium such as $CaCl_2$ and KCl enhances the milk-clotting protease production by *Rhizopus arrhizus* Fischer compared to the control , but $FeSO_4$ and $MgSO_4$ inhibit the production. In another study, **Abdel Fattah *et al*. (1984)** indicated that adding K^+ to culture medium had an adverse effect on the milk clotting activity/proteolytic activity ratio, while addition of Mg^{2+} did not significantly affect the production of the rennin-like enzyme by *Absidia cylindrospora*.

Agricultural by-products are generally used as sources of carbon and nitrogen, but the trace elements are usually added to increase production yields of various metabolites and support microbial growth, where the choice of this metallic salts has an appreciable effect according to the test micro-organism (**Tunga *et al.*, 1998**).

Effect of incubation temperature

Temperature is one of the critical parameter that has to be optimized. Fermentation carried out at 30°C was the most suitable for protease production with curdling-activity of 563.5±12.7 SU/g bran and MCA/PA 3.67 (Fig. 3). This result shows that *A. niger* FFB1 is a mesophilic strain. Increasing or lowering the incubation temperature resulted in the reduction of the activity which disappeared completely at 45°C.

Shieh *et al.* 2009 have reported that this temperature (30°C) represents the optimum for milk-clotting proteases production by *Amylomyces rouxii*, *Mucor pusillus* and *Mucor J20.* The incubation temperature affects the performance of SSF system; due to its importance in microorganisms' growth and metabolites production.

The production of protease by *A. niger* FFB1 at a lower temperature is advantageous because it leads to low rates of evaporation which avoids increasing the fermentation temperature during the incubation period. But, the reverse phenomena occur at high temperatures: the temperature rise in the fermentable mass due to a release of metabolic heat, causes drying of culture, a decrease in water activity (aw) and nutrient availability which limit aeration and induced limited growth.

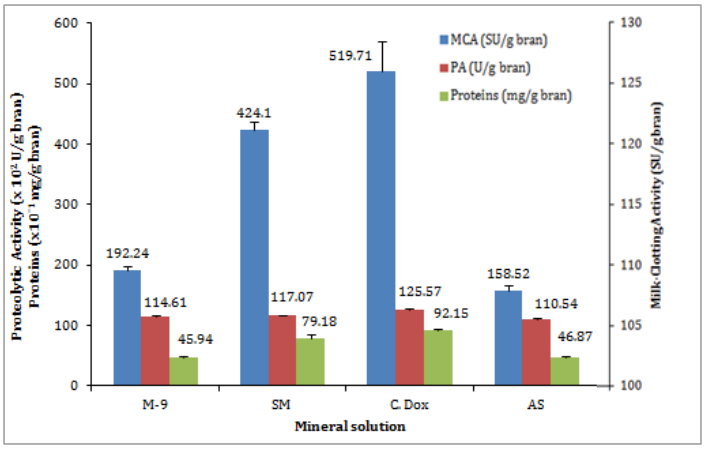

Figure 2 Effect of salts on milk-clotting protease production by *A. niger* FFB1 (Determinations were made after 4 days of stationary incubation at 28°C, pH of salt solution 5.0). Results represent the means of three essays. Error bars represent SD (Standard Deviation).

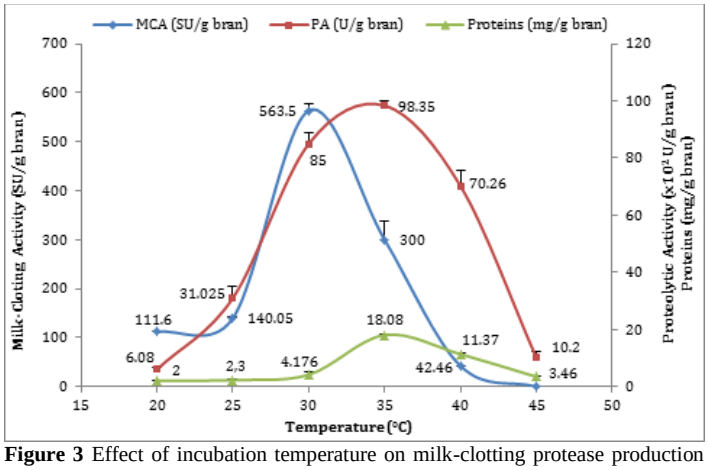

Figure 3 Effect of incubation temperature on milk-clotting protease production by *A. niger* FFB1 (use of Czapek-Dox solution). The results are the means of three essays. Error bars represent SD (Standard Deviation).

Effect of initial pH

Generally, changes of pH during SSF procedure are almost never controlled, except the initial pH of the substrate which is adjusted before inoculation (**Chutmanop *et al*., 2008**). As shown in Figure 4, protease production by *A. niger* FFB1 was maximum at pH 4 (578.8±19.434 SU/g bran). Afterward, the enzyme production decreased at all higher pH values than 4 reaching 231.9±2.507 SU/g bran at pH 7.

Similar results were found by **Singh *et al.* (1994)**, using the submerged fermentation (SmF) to produce the acidic protease of *A. niger* F2078. In contrast, **Negi and Banergee, (2010)** mentioned that the maximum protease production by *A. awamori* occurs in a medium at pH 5.5. **Vishwanatha *et al.* (2010)** found that the highest proteolytic activity of the acidic protease produced by *A. oryzae* MTTC 5341 was obtained on the same substrate (wheat bran) moistened with Czapeck-Dox solution at pH 5, while its milk-clotting activity was maximal at pH 6.3.

Our data indicate that changes in pH of the mineral medium (range 5-7) did not cause a significant difference in the enzyme activity, which can be explained by the high buffering capacity of wheat bran (also some other agro-industrial residues) and the experimental use of small amounts (**Sandhya *et al*., 2005; Chutmanop *et al*., 2008**).

The metabolic activities of microorganisms are very sensitive to pH changes, because it strongly affects many enzymatic processes and transport of various nutrients through the cell membrane ensuring the growth and the production of metabolites. These activities inevitably lead to a metabolic shift in the balance of hydrogen ions and thus the pH of the culture medium (**Elibol and Moreira, 2005; Lazim *et al*., 2009; Paranthman *et al*., 2009**).

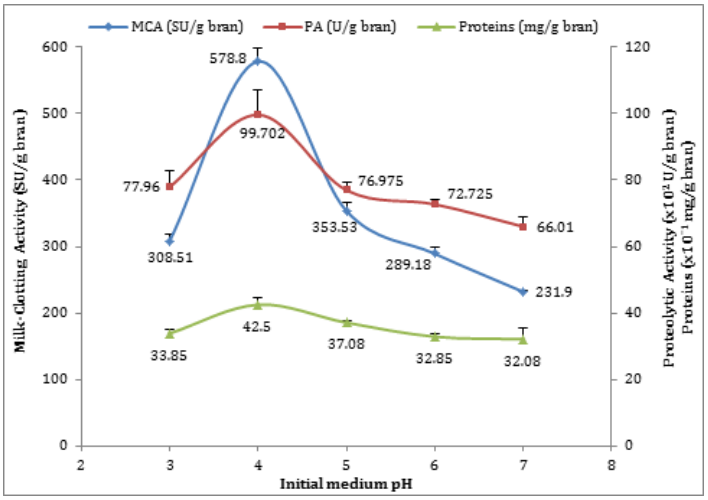

Figure 4 Influence of initial salt medium pH on milk-clotting protease production by *A. niger* FFB1 (use of Czapek-Dox solution at 30°C). The results are the means of three essays. Error bars represent SD (Standard Deviation).

Effect of moisture content of the substrate

The effect of moisture content on protease production is presented in Figure 5. Maximum production of the milk-clotting protease (747.67±17.56 SU/g bran; MCA/PA 4.6) by *A. niger* FFB1 on wheat bran was obtained at 39.2% humidity. Further, increase in this content reduced enzyme yields significantly to achieve the lower activity (70.51±3.77 SU/g bran) at a moisture level of 70.85%.

Our results are similar to those presented by **Battaglino *et al.* (1991)**, where moisture content between 35-40% was optimal for the production of protease by *A. oryzae* NRRL 2160 on rice bran. **Moosavi-Nasab *et al.* (2010)** found that the best milk-clotting activity of enzyme extracts produced by *A. niger* and *A. oryzae* are carried out at a moisture content of 30% on wheat bran. A rate lower (20%) than the values mentioned above, was used to produce protease of *Mucor circinelloides* (**Sathya *et al*., 2009**). Other reports indicated the requirement of 44.44 and 50% initial moisture content for maximum protease production by *A. oryzae* (Ozykat-1) (**Chutmanop *et al*., 2008**) and *Rhizopus microsporus* NRRL 3671 (**Sumantha *et al*., 2006**) respectively.

The content of moisture in SSF is a crucial factor that affects both microbial growth and product yields. It's varying during fermentation due to evaporation caused by metabolic heat, hydrolysis of the substrate and metabolic water production (**Sumantha *et al*., 2006; Mukherjee *et al*., 2008**).

Water is involved in cell growth, metabolic reactions, enzyme activities, transport of nutrients, extracellular metabolites and gas during SSF (spreading medium) and is also used in the formation of biological material (**Assamoi *et al*., 2009**). The low content of water causes a strong decrease in solubility of the substrate and its degree of swelling, which reduces its accessibility to the fungus, but at the same time avoiding bacterial contamination which requiring greater yields of moisture. On the other hand, at higher levels, several phenomena have been

reported: a decrease in porosity of the substrate, the loss of particle structure, the development of rigidity which causes the decrease in gas exchange (transfer of O_2 and CO_2) and increased development of aerial mycelium (**Sandhya *et al.*, 2005; Sharma *et al.*, 2008; Lazim *et al.*, 2009**).

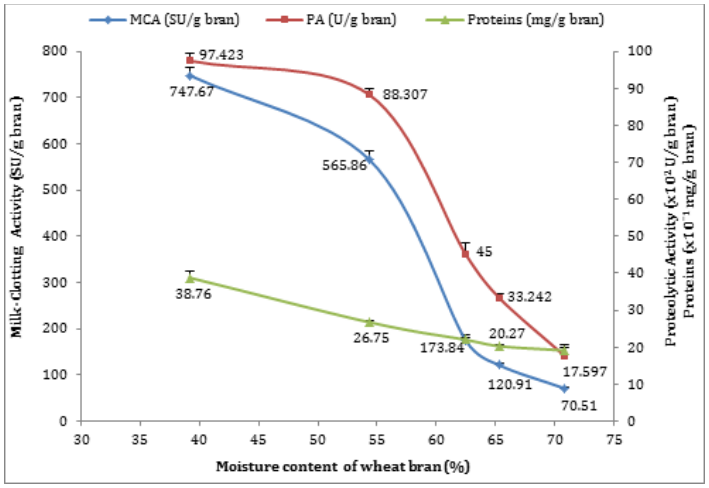

Figure 5 Effect of moisture content on milk-clotting protease production by *A. niger* FFB1 (use of Czapek-Dox solution at 30°C, initial pH 4.0). The results are the means of three essays. Error bars represent SD (Standard Deviation).

Effect of inoculum concentration

The highest enzyme activity of the crude extract produced by *A. niger* in SSF (MCA=801.12±35.12 SU/g bran; MCA/PA=4.09) was obtained when an inoculum of 10^6 spores/mL was added to the culture (Fig. 6). A higher level of inoculum decreases the milk-clotting activity of the protease to reach the lower activity (327.06±15.16 SU/g bran) when an inoculum concentration of $4.5x10^6$spores/mL was used.

A similar observation has been reported in the case of *Rhizopus oryzae* NRRL 21498 alkaline protease production (**Tunga *et al.*, 1998**). According to the results of **Ikram-Ul-Haq *et al.* (2003)**; **Pushpa and Madhava, (2010)** an inoculum concentration ranging from 0.5 to $3x10^6$spores/mL is optimal for producing the proteases of *Rhizopus oligosporus* IHS13 and *A. oryzae* by submerged fermentation and under SSF system respectively. Moreover, **Sandhya *et al.* (2005)** and **Sathya *et al.* (2009)** report that the production of proteases by *A. oryzae* NRRL 1808 and *Mucor circinelloides* is optimal when inoculum concentrations of $8x10^8$spores/mL and $3x10^9$spores/mL were applied respectively.

Inoculation of solid cultures is most often from a spore suspension which offered several advantages over vegetative cells: they remain viable longer than the mycelium, are less sensitive to external conditions and can be stored more easily. The size of inoculum is an important biological factor that determines the production of biomass in fermentation. A highly concentrated inoculum may produce excessive biomass leading to the rapid depletion of nutrients needed for rapid growth of the culture and production of metabolites, while a lower inoculum density may give insufficient biomass inducing low yields of products (**Sandhya *et al.*, 2005; Sabu *et al.*, 2006; Sharma *et al.*, 2008**).

Effect of incubation period

Milk-clotting protease production in SSF was studied over a period of 7days under the optimal conditions previously established. Milk-clotting activity was detected after 24h of incubation and culminated on day 3 reaching a maximum of 830±31.517 SU/g bran and then slightly declined till day 7 reaching 12.25±0.753 SU/g bran (Fig. 7). The same result was obtained with *A. niger* NRRL 2160 (**Moosavi-Nasab *et al.*, 2010**) and *A. oryzae* LS1 (**Shata, 2005**) on wheat bran, *Rhizopus microsporus* NRRL 3671 on rice bran (**Sumantha *et al.*, 2006**) and *A. clavatus* CCT2759 on submerged fermentation (**Tremacoldi *et al.*, 2004**).

A progressive decrease in the curdling activity has been reported with increasing incubation time, suggesting clearly the role of enzyme as a primary metabolite, produced during the mid log phase of the fungus to use nutrients (proteins) present in the solid substrate. Incubation beyond this optimal period shows a rapid decline in the yield of enzyme probably due to:

– The depletion of nutrients available to microorganisms;

– The inactivation of enzyme by other toxic metabolites released into the environment;

– The active growth of the mycelium, closely related to the incubation period and culture conditions, is essential for the production of extracellular enzymes (**Paranthman *et al.*, 2009; Lazim *et al.*, 2009**).

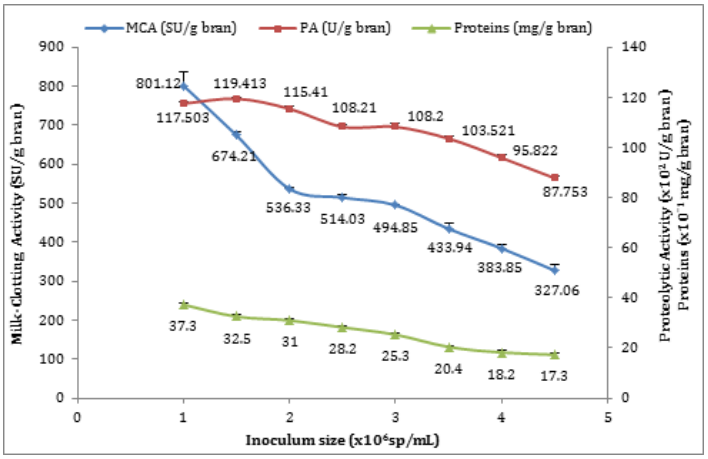

Figure 6 Influence of inoculum size on milk-clotting protease production by *A. niger* FFB1 (use of Czapek-Dox solution at 30°C, initial pH 4.0, moisture content = 39.2%). The results are the means of three essays. Error bars represent SD (Standard Deviation).

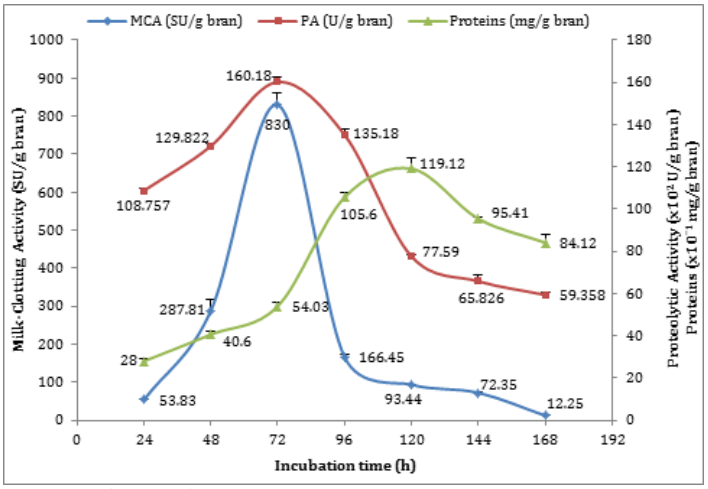

Figure 7 Influence of incubation time on milk-clotting protease production by *A. niger* FFB1 (use of Czapek-Dox solution at 30°C, initial pH 4.0, moisture content = 39.2%, inoculum size 10^6 spores/mL). The results are the means of three essays. Error bars represent SD (Standard Deviation).

Cheese making

The yields of cheeses obtained by the commercial rennet on one hand and by the protease produced by *A. niger* FFB1 on the other hand differed only by 2.8% of the product weight, but which is higher in the case of commercial rennet. This difference results from a more or less significant loss of proteins (11%) and fat (4%) during the draining, induced especially by the proteolytic activity of the crude extract produced by *A. niger* (Table 1).

The sensory characteristics of the experimental cheese made with the crude extract of *A. niger* were quite similar to cheese prepared with the commercial rennet. The good taste and a granular texture were observed by all tasters: no bitterness was reported. The smell of raw milk was presented in the two cheeses with a strong odour in the cheese made with fungal crude extract, which presents also the best texture then commercial rennet.

Table 1 *Mean composition of cheeses*

Cheese	*Cheese physicochemical parameters*				
	Dry extract content (%)	**Fat matter (% of weight)**	**pH**	**Moisture (%)**	**Product weight (g/l)**
Cheese made with commercial rennet	44.9	21	6.28	55.1	**128**
Cheese made with *A. niger* FFB1	47.9	19	6.16	52.1	**100**

CONCLUSION

The aim of our study was to search the optimum culture conditions for the milk-clotting enzyme production by *A. niger* FFB1 in solid-state fermentation. One-factor-at-a-time methodology was used to optimize the different factors and to assess their effects on enzyme production and its activities. The results showed that the crude enzymatic extract, obtained from the culture of *A. niger* using wheat bran as substrate was characterized by a maximum milk-clotting activity of 830SU/g bran and a ratio MCA/PA of about 4.25 which are markedly affected by changing medium conditions. The optimum levels of factors were incubation temperature of 30°C, spores concentration of 10^6spores/mL, moisture content of substrate of 39.2% which was obtained by using Czapek-Dox solution (pH 4) and a fermentation time of 72hours.

Compared to researches that has been done on the replacement of animal rennet by various substitutes, we can concluded that our results are very encouraging and pave the way to test the ability of this crude extract and the purified enzyme in the production of other types of cheese that require a ripening step to study their influence on this step.

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