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 cheap 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 (105 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 clotting enzyme, protease, optimized for milk 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 (105 spores/mL), incubation time (72 hours), and moisture content of solid substrate (39.2%) adjusted suitably with mineral solution (Czapek-Dox) of pH 4.

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 Phe105-Met108 bond which results in the release of hydrophilic glycopeptide (caseminoacarpropeptide) 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 (αs1, αs2, β and γ) occurs under the influence of Ca2+ 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, solving pollution problems and reduce the capital costs of process (Vishwanatha et al., 2010; Sathiya 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 (Agraval et al., 2005; Sandhya et al., 2008). 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 2x106 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 Czapek-Dox agar medium containing: Saccharose (30 g/L), NaNO₃ (3 g/L), KH₂PO₄ (1 g/L), MgSO₄·7H₂O (0.5 g/L), KCl (0.5 g/L), FeSO₄·7H₂O (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: NH₄NO₃ (5 g/L), KH₂PO₄ (2 g/L), MgSO₄·7H₂O (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 determine 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 other 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: NaNO₃ (2.5 g/L); KH₂PO₄ (1 g/L); MgSO₄·7H₂O (0.5 g/L); KCl (0.5 g/L)
- M-9 solution: NH₄NO₃ (3 g/L); KH₂PO₄ (1 g/L); MgSO₄·7H₂O (1 g/L); FeSO₄·7H₂O (0.01 g/L) (Tunga et al., 1998)
- SM solution: NH₄NO₃ (5 g/L); KH₂PO₄ (2 g/L); MgSO₄·7H₂O (1 g/L); NaCl (1 g/L) (Sumantha et al., 2005; Paranthaman 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 and 40°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 5x10⁶ 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/w). 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 Soskht 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: 

\[ \text{MCA (SU/mL)} = \frac{24000 \times T \times S}{E} \]

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 filtered to remove the precipitate, then to 1mL of supernatant, 2.5mL of 2% Na₂CO₃ and 0.5mL of diluted Folin Cioclațu’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). CHRY-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% NaCl) 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 proteolytic 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/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 Czapek-Dox solution added to wheat bran ensures increasing in production of enzyme with milk-clotting activity 519.71±49.47 SU/g bran probably related to the presence of KCl in the

Maximum alkaline protease activities of Rhizopus oryzae NRRL 21498, Bauveria felina and Aspergillus oryzae NCIM 649 have been reported using M-9 solution

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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 cyliospora.

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 salt 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 Amylomonas rouxi, Mucor pusillus and Mucor L20. 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 culture medium resulted in the reduction of the activity which disappeared completely at 45°C.

**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 fermentations (SFM) to produce the acidic protease of A. niger F2078. In contrast, Negi and Banerjee, (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 Czapek-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 (Elbılı and Moreira, 2005; Lazim et al., 2009; Paranthaman et al., 2009).

**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±231.9) 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±13.77 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 60% of initial moisture content and maximum protease production by A. oryzae (Ozyakat-1) (Chutmanop et al., 2008) and Rhizopus microsporus NRRL 3671 (Sumanta 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 (Sumanta 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\textsubscript{2} and CO\textsubscript{2}) and increased development of aerial mycelium (Sandhya et al., 2005; Sharma et al., 2008; Lazim et al., 2009).

**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\textsuperscript{5} 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\textsuperscript{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-U-Haq et al. (2003), Pushpa and Madhava, (2010) an inoculum concentration ranging from 0.5 to 3x10\textsuperscript{5} 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 Sathyaa et al. (2009) report that the production of proteases by A. oryzae NRRL 1808 and Mucor circinelloides is optimal when inoculum concentrations of 8x10\textsuperscript{5} spores/mL and 3x10\textsuperscript{5} 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 size may give insufficient biomass for 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 72days under the optimal conditions previously established. Milk-clotting activity was detected after 24h of incubation and culminated on day 3 reaching 12.25±0.753 SU/g bran (Fig. 7). The same result was obtained with A. niger NRRL 2160 (Moosavi-Nasabi et al., 2010) and A. oryzae LSI (Shata, 2005) on wheat bran, Rhizopus microsorus NRRL 3671 on rice bran (Sumantaha 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 (Paranthaman et al., 2009; Lazim et al., 2009).

![Figure 5 Effect of moisture content on milk-clotting protease production by A. niger FFB1](image1)

![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). The results are the means of three essays. Error bars represent SD (Standard Deviation).](image2)

![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%). The results are the means of three essays. Error bars represent SD (Standard Deviation).](image3)

### Table 1 Mean composition of cheeses

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

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.

**Cheese physicochemical parameters**

- **Dry extract content (%)**: 44.9 - 47.9
- **Fat matter (% of weight)**: 21 - 19
- **pH**: 6.28 - 6.16
- **Moisture (%)**: 55.1 - 52.1
- **Product weight (g)**: 128 - 100
CONCLUSION

The aim of our study was to search the optimum culture conditions for the milk-clotting enzyme production by A. niger FF81 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 4.23 which are markedly affected by changing medium conditions. The optimum levels of factors were incubation temperature of 30°C, spores concentration of 10^6 spores/mL, moisture content of substrate of 39.2% which was obtained by using Czapek-Dox solution (pH 4) and a fermentation time of 72 hours.

Compared to researches that has been done on the replacement of animal rennet by various microorganisms, 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.

REFERENCES

ABDEL FATTAH, A. F., ISMAIL, A. M. S., EL-ASRAR, S. A. 1984. Production of rennin-like enzyme by Absidia cylindrospora. Journal of Agricultural Wastes, 11, 125-131. http://dx.doi.org/10.1016/S0144-6075(97)80072-4

AGRAWAL, D., PATIDAR, P., BANERJEE, T., PATIL, S. 2005. Alkaline protease production by a soil isolate of Beauveria fujinensis under SSF condition: parameter optimization and application to soy protein hydrolysis. Process Biochemistry, 40, 1131-1136. http://dx.doi.org/10.1016/j.procbio.2004.03.006

ANSON, M. L. 1938. The estimation of pepsin, trypsin, papain and cathepsin with the aid of a globin and General Physiology, 19, 446-459. http://dx.doi.org/10.1016/0278-1000(38)90057-7

AMARGILLO, S. 1986. Recueil de Normes Françaises: Contrôle de la Qualité des Produits laitiers, Analyses Physiques et Chimiques. 1030 p. 3rd eddition. ITSV Publications, Recueil de Normes Français.

BENDARY, M. A., MOHARAM MAYSA, E., ALI THANAA, H. 2007. Production of proteases by a locally isolated mould culture under lab state fermentation. Methods in enzymology, 459. Available online at http://www.sciencedirect.com/science/article/pii/0076687976905273

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Annals of Biochemistry, 72, 248-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3

CHUTMANOP, J., CHUICHULCHERM, S., CHISTI, Y., SRINOPHAKUN P. 1989. Production and application of amylase by industrial Wastes. Journal of Agricultural Sciences, 3(8), 199-206. http://dx.doi.org/10.1016/0021-8616(89)90047-0

EL-BENDARY, M. A., MOHARAM MAYSAA, E., ALI THAHAA, H. 2007. Purification and characterization of milk-clotting enzyme production by Bacillus sphaericus. Journal of Applied Sciences Research, 3(8), 695-699. www.sciencedirect.com/science/article/pii/S1816795407695-699.pdf

EL-BOL, M., MOREIRA, A. R. 2005. Optimizing some factors affecting alkaline protease production by a marine bacterium Tethrocinobacter turnarium under solid-state fermentation. Process Biochemistry, 40,1951-1956. http://dx.doi.org/10.1016/j.procbio.2004.07.010

FAZIOUANE, YAIMI, F., MECHAKRA, A., ABDELLAOUI, R., NOUANI, A., MAGAGI DAGA, S., MAROU ALZOUMA, A., GAIS, S., PENNINCKX M. J. 2010. Characterization and cause-making properties of rennet-like enzyme produced by a local Algerian isolate of Aspergillus niger. Food Biotechnology, 24, 258-269. http://dx.doi.org/10.1080/02698251003478714

FOX, P. F. 1999: Cheese: chemistry, physics and microbiology: general aspects Volume I. 2nd Edition An Aspen Publication, New York, 601p. ISBN 0-8342-1338-9

FOX, P. F., MCSWEENEY, P. L., COGAN, T. M., GUINNE, T. P. 2004. Cheese: chemistry, physics and microbiology: general aspects Volume I. 3rd Edition Elsevier Academic Press, United Kingdom, 640p. ISBN 0-1226-3652-X

KUMAR, D., JAIN, V. 2003. Production of proteases by a locally isolated mould culture under lab conditions. Biotechnology, 2, 30-36. http://dx.doi.org/10.3923/biotech.2003.30.36
TREMACOLDI, C. R., WATANABE, N. K., CARMONA, E. C. 2004. Production of extracellular acid proteases by Aspergillus clavatus. *World Journal of Microbiology and Biotechnology*, 20, 639-642. [http://dx.doi.org/10.1023/B:WIBI.0000043194.21060.c1](http://dx.doi.org/10.1023/B:WIBI.0000043194.21060.c1)

TUNGA, R., BANERJEE, R., BHATTACHARYA, B. C. 1998. Optimizing some factors affecting protease production under solid-state fermentation. *Bioprocess Engineering*, 19, 187-190. [http://dx.doi.org/10.1007/BF00900504](http://dx.doi.org/10.1007/BF00900504)

VISHWANATHA, K. S., RAO APPU, A. G., Sigh S. A. 2010. Acid protease production by solid-state fermentation using *Aspergillus oryzae* MTCC 5341: optimization of process parameters. *Journal Industrial of Microbiology and Biotechnology*, 37, 129-138. [http://dx.doi.org/10.1007/s10295-009-0654-4](http://dx.doi.org/10.1007/s10295-009-0654-4)

WANG, J., WAN, W. 2009. Experimental design methods for fermentation hydrogen production: A review. *International Journal of Hydrogen Energy*, 34, 235-244. [http://dx.doi.org/10.1016/j.ijhydene.2008.10.008](http://dx.doi.org/10.1016/j.ijhydene.2008.10.008)

WU, J., CHEN, H., CHEN, W. 2008. Fermentation parameter and partial biochemical characterization of milk clotting enzyme from Chinese distiller’s yeast, *Annals of Microbiology*, 58(4), 717-722. [http://dx.doi.org/10.1007/BF03175580](http://dx.doi.org/10.1007/BF03175580)