

Aspartic proteinases from *Mucor* spp. in cheese manufacturing

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Abstract Filamentous fungi belonging to the order of Mucorales are well known as producers of aspartic proteinases depicting milk-clotting activity. The biosynthesis level, the biochemical characteristics, and the technological properties of the resulting proteinases are affected by the producer strain and the mode of cultivation. While the milk-clotting enzymes produced by the *Rhizomucor* spp. have been extensively studied in the past, much less is known on the properties and potential applications of the aspartic proteinases obtained for *Mucor* spp. Indeed, several *Mucor* spp. strains have been reported as a potential source of milk-clotting enzymes having unique technological properties. Both submerged fermentation and solid substrate cultivation are proven alternatives for the production of *Mucor* spp. aspartic proteinases. This review provides an overview on the bioprocessing routes to obtain large amounts of these enzymes, on their structural characteristics as related to their functional properties, and on their industrial applications with focus on cheese manufacturing.

Keywords *Mucor* spp. proteinase · Aspartic proteinase · Acid protease · Milk-clotting enzymes

Aspartic proteinases—an introduction

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteinases are classified as hydrolyses (group 3) and within the subgroup 4, i.e., those which are able to hydrolyze peptide bonds. They have been divided into six mechanistic classes including cysteine proteinases, serine proteinases, aspartic proteinases, metalloproteinases, threonine proteinases, and unknown type proteinases. The threonine proteinase group is the latest that has been described (Seemuller et al. 1995). Each class has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site. Proteinases can also be subdivided into two major groups based on their ability to cleave N- or C-terminal peptide bonds (exopeptidase) or internal peptide bonds (endopeptidase). Further, while aminopeptidase cleave N-terminal peptide linkages, carboxypeptidases cleave the C-terminal peptide bonds.

Aspartic proteinases (EC 3.4.23), also known as acid proteinases or aspartyl proteinases, are endopeptidases having two aspartic acid residues that are critical for their catalytic activity (Asp32 and Asp215, pepsin numbering) within their active site. Most aspartic proteinases show maximal activity at low pH values (~3–5). They are acidic in nature, having isoelectric points in the range of 3 to 4.5. They are inhibited by pepstatin A, a hexapeptide from *Streptomyces* that contains two statin residues, an unusual amino acid.

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Besides, they are also inhibited by diazoketone compounds such as diazoacetyl-DL-norleucine methyl ester and 1,2-epoxy-3-(*p*-nitrophenoxy) propane in the presence of copper ions (Rao et al. 1998). Aspartic proteinases have been grouped into three families, namely pepsin (A₁), retropepsin (A₂), and enzymes from pararetroviruses (A₃). Structurally, most aspartic proteinases belong to A₁ pepsin family. Like other pepsin-like enzymes, aspartic proteinases are synthesized as pre-proenzymes (zymogen). After cleavage of the signal peptide, the proenzyme is secreted and autocatalytically activated (Davies 1990). Generally, the active enzyme consists of a single peptide chain of about 320–360 amino acid residues and has a molecular mass of 32–36 kDa. X-ray crystallographic analyses of various aspartic proteinases showed that they are composed of β -strand secondary structures arranged in a bilobal conformation (Aguilar et al. 1997; Claverie-Martin and Vega-Hernandez 2007). The two lobes are homologous of each other and have most likely evolved by gene duplication. The catalytic center is located between the two lobes and contains a pair of aspartic acid residues, one in each lobe, that are essential for the catalytic activity. These aspartate residues activate a water molecule that mediates the nucleophilic attack on the substrate peptide bond. Andreeva and Rumsh (2001) have found that another water molecule plays an essential role in the formation of a chain of hydrogen bonds that determines the substrate binding. The catalytic site is large enough to accommodate at least seven residues of the polypeptide substrate.

Aspartic proteinases are mainly present in animal cells, plants, molds, and yeasts but seldom in bacteria (Sumantha et al. 2006). They have been linked to a variety of physiological functions including mammalian digestion (e.g., chymosin, pepsin). Microbial aspartic proteinases can be divided into two groups: (a) pepsin-like enzymes produced from *Aspergillus* spp. (Vishwanatha et al. 2009) and *Rhizopus* spp. (Kumar et al. 2005) and (b) rennin-like enzymes produced by *Mucor* spp. (Fraile et al. 1981; Venera et al. 1997; Fernandez-Lahore et al. 1999; Neelakantan et al. 1999; Andrade et al. 2002; Machalinski et al. 2006), *Rhizomucor* spp. (Thakur et al. 1990; Escobar and Barnett 1993; Silveira et al. 2005; De Lima et al. 2008), and *Endothia parasitica* (Sardinas 1968).

Aspartic proteinases were the first type of enzymes known to mankind, the first proteinase type described, the second protein crystallized, and also the target for numerous early investigations by the pioneers of modern enzymology and protein chemistry (Szecsi 1992). For example, bovine chymosin—in the form of calf rennet—has been used for millennia in cheese making, dating back to approximately 6000 BC.

Selective and high-quality biosynthesis of aspartic proteinases from *Mucor* spp.

Mucorales are ubiquitous, morphologically simple terrestrial fungi. This order comprises *Rhizopus*, *Mucor*, *Rhizomucor*, *Absidia*, and *Cunninghamella* as members. Both *Mucor* spp. and *Rhizomucor* spp. are well-known producers of aspartic proteinases with high milk-clotting activity. These two species distinguish from each other in terms of some morphological traits and the optimum cultivation temperature, *Rhizomucor* spp. being of a thermophilic nature. *Rhizomucor pusillus* and *Rhizomucor miehei* are a common industrial source of microbial rennets. Fraile et al. (1981) claimed that the utilization of commercially available heat-stable enzymes in cheese manufacturing may be limited due to undesirable changes in the organoleptic quality of the final product. It is interesting to note that attempts have been made to reduce the thermostability of microbial rennets by genetic engineering approaches. For example, Yamashita et al. (1994) prepared, by site-directed mutagenesis, a *R. pusillus* mutant gene which expressed an active enzyme having a much lower thermostability than the wild-type enzyme. The mentioned recombinant proteinase was expressed in yeast. Table 1 summarizes the characteristics of some microbial aspartic proteinases.

Several reports described the efficient biosynthesis of aspartic proteinases by *Mucor* spp. strains (Belyauskaite et al. 1980; Fraile et al. 1981; Handel and Fraile 1984; Fernandez-Lahore et al. 1998; Andrade et al. 2002). Fraile et al. (1978) performed a pioneering study on the ability of *Mucor* spp. strains to synthesize milk-clotting enzymes. A collection of 41 strains were tested out of which 28 strains turned out to be active producers of such proteinases. Some of the producer strains were identified as *Mucor racemosus*, *Mucor fragilis*, *Mucor mucedo*, *Mucor spinosus*, *Mucor rouxii*, *Mucor varians*, *Mucor bacilliformis*, and *Rhizomucor hiemalis*. Further and more detailed studies focused on the biosynthesis of aspartic proteinases by *M. bacilliformis* (Arecas et al. 1992), *M. varians* var *Pispek* (Bernardinelli et al. 1983), *M. mucedo* (Handel and Fraile 1984), and *Mucor renninus* (Belyauskaite et al. 1980). Tubesha and Al-Delaimy (2003) studied 20 *Mucor* spp. environmental isolates originating from Jordan. The highest milk-clotting activity was obtained from the so-called *Mucor* J120 in a solid cultivation medium containing wheat bran and lentil straw. Although no complete information on the taxonomical classification of the isolates at the species level was provided, this study confirms the potential of *Mucor* spp. for the biosynthesis of aspartic proteinases. Other authors (Alves et al. 2005) have observed the biosynthesis of extracellular proteinases by *M. racemosus* f. *chibinensis* and also by *Mucor circinelloides* f. *circinelloides*, *M. circinelloides* f. *griseo-cyanus*, *M. circinelloides* f. *janssenii*, *M. circinelloides* f. *lusitanicus*, *Mucor genevensis*,

Table 1 Characteristics of some microbial aspartic proteinases

Fungi	Molecular mass (kDa)	Optimum temperature (°C)	Optimum pH	pI	Reference
<i>Rhizomucor pusillus</i>	38.5	55	5.6	3.9	Etoh et al. (1979)
<i>Rhizomucor miehei</i>	20–43	58–62	5.6*	4.1	Preetha and Boopathy (1994), Etoh et al. (1979)
<i>Mucor hiemalis</i>	–	–	3.0–3.5	–	Wang (1967)
<i>Mucor renninus</i>	35	–	3.5	5.1	Belyauskaite et al. (1980)
<i>Mucor varians</i> Pispek	–	–	3.0	–	Bernardinelli et al. (1983)
<i>Mucor rouxii</i>	16	50	4.0	–	Diaz and Ruiz Herrera (1987)
<i>Mucor bacilliformis</i>	32–35	45	3.0–3.5	5.2	Areces et al. (1992), Venera et al. (1997), Machalinski et al. (2006)
<i>Mucor circinelloides</i>	33	–	3.0–3.5	4.2	Fernandez-Lahore et al. (1999)
<i>Rhizopus oryzae</i>	34	60	5.5	–	Kumar et al. (2005)
<i>Aspergillus oryzae</i>	47	55	3.2	–	Vishwanatha et al. (2009)
<i>Endothia parasitica</i>	34–39	–	4.5	5.5	Sardinas (1968)
<i>Penicillium duponti</i>	41	55	2.5	3.9	Hashimoto et al. (1973)

*Enzyme having milk clotting activity (The microorganism produces different kinds of aspartic proteinases)

Mucor hiemalis f. *hiemalis*, *M. hiemalis* f. *luteus*, *Mucor piriformis* f. *piriformis*, *M. piriformis* f. *nanus*, *M. racemosus* f. *chibinensis*, *Mucor subtilissimus*, *Mucor variosporus*, and *Mucor carbonaceus*. Aspartic proteinases may have well contributed, to a larger or minor extent, to the total proteolytic activity observed in the mentioned microbial strains. Further and more focused studies are required to fully clarify this point.

Since *Mucor* spp. are mesophilic microorganisms, it is likely that they produce aspartic proteinases which are more sensitive to temperature than those derived from *Rhizomucor* spp. This concept has been proven experimentally: the *R. pusillus* enzyme retains 71% of its original activity when heated for 10 min at 55 °C while, under similar conditions, the calf rennet and the *M. bacilliformis* aspartic proteinase retain 31% and 10% of the initial milk-clotting activity, respectively (Fraile et al. 1981). Fernandez-Lahore et al. (1999) have shown that a *M. circinelloides* strain (M-105) produces a wild-type enzyme with very promising characteristics, being even less heat stable than the enzyme obtained from *M. bacilliformis* cultures.

In addition to the unfavorable properties of the *Rhizomucor* spp. milk-clotting enzymes, these microbial strains are usually able to produce a range of other proteases and/or enzymes, e.g., lipases or amylases. In some cases, the removal of these interfering activities can be performed by simpler methods like fractional precipitation or by negative adsorption. For example, Thakur et al. (1993) used different adsorbents including different clay minerals for the removal of unspecific proteinases and lipases from the crude enzyme extract obtained from the solid-state cultivation of *R. miehei*. Inactivation of undesirable enzymes by limited exposure to extreme pH or temperature could work under specific circumstances. On the contrary, several studies on

the production of *Mucor* spp. aspartic proteases showed the absence of interfering enzyme activities and the absence of unwanted secondary metabolites, like antibiotics or aflatoxins in the final preparations obtained from the cultures.

Diaz and Ruiz Herrera (1987) reported on the purification and characteristics of an intracellular proteinase, obtained from washed mycelia of *M. rouxii* that was grown by submerged fermentation (SmF) in shaking flasks. Interestingly, a large proportion (89%) of the total proteolytic activity found in disrupted mycelia crude extract was inhibited by pepstatin A, which indicates that aspartic proteinases are mainly responsible for such activity. This is not a common finding since the microbial intracellular space usually contains a large number of different types of proteases. Therefore, the biosynthesis of aspartic proteinases by *Mucor* spp. may not be limited to the secreted enzymes but may also include a large amount of proteinases which are located inside the cell.

Summarizing, the evidence gathered from literature may indicate that several *Mucor* spp. strains could be selective producers of aspartic proteinases that mainly depict milk-clotting activity and that those enzymes also show heat sensitivity and industrially desirable characteristic during cheese manufacturing.

Production of *Mucor* spp. aspartic proteinases by submerged fermentation

SmF are usually carried out with a substrate, which is either dissolved or remains suspended in an aqueous medium. The utilization of different substrates can affect proteinase production. A common observation is that while simple substrates, such as casein and gelatin, yield low enzyme

activity units, more complex substrates, such as soybean meal and wheat bran, result in higher proteinase activities (Boer and Peralta 1999; Sumantha et al. 2006). On the other hand, it has been frequently described that, in a defined medium, a protein source must be present for the enzyme to be produced (Andrade et al. 2002).

M. mucedo is unique among other *Mucor* spp. strains in its ability to produce a milk-clotting enzyme in submerged fermentation as opposed to solid-state fermentation (SSF). Handel and Fraile (1984) investigated the production of milk-clotting enzymes using *M. mucedo* in aerated submerged culture. Casein and cheese whey were used as sole organic nitrogen sources. The highest enzyme activity was obtained at casein concentration between 0.5% and 0.7% (w/v). The effect of different glucose concentrations (0%, 0.5%, 1%, and 2% w/v) on the enzyme synthesis was studied; the optimum concentration of glucose was 1%. The effect of cations (Fe^{2+} , Cu^{2+} , Mn^{2+} , and Zn^{2+}) and anions (SO_4^{2-} and PO_4^{2-}) on the production of enzyme was also observed. In the absence of cations, production was depressed by 40% while the lack of each anion separately exhibited little effect on enzyme synthesis. Anions showed a more pronounced effect on enzyme production in a concentration-dependent manner. However, a note of caution has to be raised when analyzing the mentioned results: Belyauskaite et al. (1980) investigated the effect of different metal ions (Ca^{2+} , Mg^{2+} , Cu^{2+} , and Co^{2+}) on the activation of the proteolytic enzymes produced by *M. renninus* under submerged fermentation conditions. They found that Cu^{2+} strongly activated such enzymes in vitro. Summarizing, the results observed by Handel and Fraile (1984) could be a combination of microbial physiology-related and enzyme-activation-related effects.

Recently, studies on the production of milk-clotting enzymes by *M. mucedo* DSM 809 in submerged fermentation were conducted by Yegin et al. (2010). The maximum activity was observed after 48 h of cultivation at 24 °C in Erlenmeyer flasks. The optimized initial pH and shaking speed for enzyme production were 4.5 and 220 rpm, respectively. Glucose at a concentration of 1% (w/v) was the best carbon source for the production of enzyme among the carbohydrates examined (glucose, fructose, lactose, and maltodextrin). On the other hand, casein at a concentration of 0.5% (w/v) was the selected nitrogen source in the media formulation. Under optimized conditions, enzyme levels reached 130 U/ml of fermentation broth. The inoculum type and size have also affected biomass production and the biosynthesis of the enzyme; the maximum activity was obtained with spores at a total load of 6.0×10^5 spores per flask. Further studies are needed in order to elucidate the nature and the characteristics of the enzyme (s) produced and to increase production levels in bioreactors.

Information on the submerged cultivation and the production of aspartic proteinases by *Mucor* spp. other than *M. mucedo* is almost inexistent. However, data obtained on the production of milk-clotting enzymes by *Rhizomucor* spp. are abundant and can provide some additional and useful guideline in order to understand *Mucor* spp. behavior. Seker et al. (1998) and Beyenal et al. (1999), who worked on the production of the aspartic proteinase from *R. miehei*, pointed out that a high glucose concentration in the fermentation media inhibited enzyme activity, as it stimulated biomass rather than rennin production. Silveira et al. (2005) also investigated the effect of carbon and nitrogen sources on rennin production by *R. miehei* in submerged fermentation. In this case, an optimized glucose concentration (18 g/l) yielded maximum enzyme activity levels, but product inhibition was detected when the glucose concentration increased to 25 g/l. High enzyme activity (667–923 U/ml) was obtained when corn step liquor and Proflo[®] were utilized as nitrogen source. Another study by De Lima et al. (2008) investigated the growth pattern of *R. miehei* NRRL 3420 in SmF. The production of the milk-clotting enzyme was studied—employing statistical design methods—as a function of lactose (from cheese whey) and glucose amounts, which were added as carbon sources. The highest enzyme activity measured was 1,200 U/ml of fermentation broth. This level was obtained with a combination of 20 g/l of glucose and 4 g/l of casein at pH 4.0. When compared to glucose, lactose was considered as an inferior source of carbon for the production of rennin. As expected for a primary product of metabolism, the enzyme production was associated with cell growth. These results, taken as a whole, indicated a positive effect of glucose and other carbohydrates—up to a certain threshold—on enzyme synthesis whereas casein and other nitrogen sources induced proteinase production in *Rhizomucor* spp.

Additional information can be gathered from Andrade et al. (2002), who studied the production of extracellular proteinases from *M. circinelloides* using different concentrations of D-glucose (40, 60, and 80 g/l) as a substrate. Although highest biomass production was obtained in a medium containing D-glucose at a concentration of 80 g/l, the maximum enzyme production was found when the substrate concentration was reduced to 40 g/l. According to the mentioned authors, glucose was found to be a reasonably good substrate for enzyme production, especially when used at the lowest concentration these authors have tested. However, it has been also noticed that a lack of such substrate resulted in a dramatic decrease in the enzyme production. These results suggested that *M. circinelloides* could be a promising strain for aspartic proteinase production. Parallelisms can be found between studies performed on some *Rhizomucor*

spp. and *Mucor* spp. strains when cultivated in submerged fermentation.

The potential for scale-up of the production of aspartic proteinases from *Mucor* spp. is clear from the bioprocess setup mentioned by Belyauskaite et al. (1980). These authors run a submerged fermentation at the 1.5-m³ scale, utilizing media composed of 3% maize starch, 1.5% casein, 0.2% calcium chloride, and 0.05% mono-potassium phosphate (pH 5.5). The sterile broth was inoculated with 3% *M. renninus* mycelia. Other cultivation parameters were temperature of 30 °C, aeration rate of 0.85 m³/m³ per minute, and stirring velocity of 200 rpm. Unfortunately, no information on total milk-clotting enzyme yields was reported in this study.

Production of *Mucor* spp. aspartic proteinases by solid-state fermentation

SSF involves all those processes which utilize water-insoluble material for microbial growth in the absence of free water. These conditions tend to favor the growth of filamentous fungi. The advantages of SSF include simplicity, lower production costs, high enzyme yields, and low wastewater output. An additional advantage is the facilitated recovery of the products, e.g., an enzyme can be leached out in a concentrated solution. Moreover, SSF helps to prevent phenomena that adversely influence the biosynthesis of aspartic proteinase as in the cases of catabolite repression by glucose or the case of biosynthesis inhibition by amino acids and ammonia (Fernandez-Lahore et al. 1998). Several types of aspartic proteinases have been successfully produced by this technique (Preetha and Boopathy 1994; Pandey et al. 2000; Kumar et al. 2005).

Previous studies may indicate that *Mucor* spp. strains are good aspartic proteinase(s) producers in solid cultures. For example, Higashio and Yoshioka (1982) screened several fungal strains and found that *M. racemosus* produced a milk-clotting enzyme reaching 6,500 U/g of solid substrate in SSF systems, which would correspond to a crude extract containing 600–1,000 U/ml. As a comparison, the same strain was able to reach enzyme activity between 600 and 800 U/ml of culture supernatant after submerged fermentation. Moreover, some authors have reported that the quality of the final milk-clotting enzyme preparations obtained via SSF is improved when compared to those obtained by the SmF technique (Thakur et al. 1990).

As mentioned above, *Mucor* spp. strains can produce milk-clotting enzyme(s) under SSF conditions, typically yielding 5,000 U/g of solid substrate after 5–6 days of cultivation at moderate temperatures. The solid substrate mixture, the initial moisture content, the substrate pH, the substrate porosity, the cultivation temperature, the presence

of added nutrients, and the inoculum size are critical factors concerning process yields. Highest productivity can be obtained with selected strains at starting moisture level of 90% (dry weight basis), at 24–28 °C, at initial acidic pH values, and by utilizing wheat bran as substrate (Fraile et al. 1978, 1981; Fernandez-Lahore et al. 1997). Other authors have reported that enzyme production in a supplemented wheat bran mixture was optimal by cultivating at 30 °C and harvesting the fermented mass after 3–4 days (Tubeshia and Al-Delaimy 2003). All the enzymes produced by *Mucor* spp. in SSF have shown remarkable heat sensitivity.

Fraile et al (1981) studied the production of milk-clotting enzyme from a *M. bacilliformis* strain (M-88) on wheat bran medium and reported on some properties of the crude extract obtained after extraction of the fermented mass with water. The peak of enzyme production was observed after 85 h of cultivation, reaching milk-clotting activity levels within the 3,000–4,000 U/g range. The crude enzyme had an optimal activity at pH 4.0, which is very close to the pH optima of calf rennet (pH 3.7). It was also mentioned that the enzyme was stable between pH 3.0 and 6.0, as it was observed for calf rennet and the *R. miehei* proteinase. Similarly, Fernandez-Lahore et al. (1997) tested several media formulations in order to enhance the production of aspartic proteinase by *M. bacilliformis*. Durum wheat bran, bread wheat bran, rice bran, sunflower hull, and different mixtures of the substrates thereof were tested. The best results were obtained with durum wheat bran as a sole substrate. Further enrichment of the same by addition of glucose (50 mg/g), wheat flour (10% w/w), skim milk (1% w/w), or a cocktail of mineral ions did not improve the production of the enzyme. The optimized fermentation system—based on wheat bran wetted to 120% (wet basis) with 0.2 M chloridric acid solution and inoculated with 5 × 10⁵ spores per gram—produced a maximum milk-clotting activity of 7,500 U/g after 72 h of cultivation at 24 °C. Further studies by Lareo et al. (2006) on this strain were conducted on a synthetic solid substrate. Growth and sporulation of *M. bacilliformis* on inert polyurethane foam at varying water activity values and nutrient concentrations were investigated. Maximum biomass (0.59 g/g_{inert support}) and spore production (6.0 × 10⁸ spores per gram_{inert support}) were obtained at 90% (w/w) water content. The biomass, CO₂ production, and glucose consumption profiles of *M. bacilliformis* were modeled, and estimates of the corresponding kinetic parameters were obtained. Maximum specific growth rates (μ_{\max}), using the logistic and the exponential models, were determined from biomass data as 0.12 and 0.40 h⁻¹, respectively. The same parameter was determined from CO₂ evolution data as 0.14 and 0.73 h⁻¹ depending on the model utilized to fit such data, as before. Although the logistic model fitted the CO₂ data well, it did not predict the active biomass levels after carbon source

consumption, and therefore, this model can estimate yield coefficients (Y_x/CO_2) and maintenance coefficients (mCO_2) with some deviations. Such parameters are useful for design and control purposes in SSF systems (Mitchell et al. 2004).

Bernardinelli et al. (1983) studied the production of the milk-clotting enzyme from *M. varians* Pispék under SSF on wheat bran. Milk-clotting activity of up to 4,000 U/g of solid substrate was achieved. The enzyme was easily leached out from the fermented mass with water as extractant and subsequently recovered by precipitation with ammonium sulfate or organic solvents (ethanol and acetone). The crude enzyme showed an optimum pH \approx 3.0 when casein and denatured hemoglobin were used as assay substrates. This acid proteinase was fairly unstable at pH values higher than 7.0 and temperature values higher than 45 °C. Antibiotic activity was absent, and interfering enzymatic activities, i.e., amylase and lipase were negligible or within the range encountered in other commercial products.

Fernandez-Lahore et al. (1999) reported the production, purification, and partial characterization of an aspartic proteinase derived from *M. circinelloides* (M-105). Durum wheat bran was utilized as solid substrate. These authors reported that the biosynthesis of the enzyme, measured via a milk-clotting assay, reached its maximum on the fourth day of cultivation. At the peak of production, an activity level of 7,100 U/g was observed. Recently, Sathya et al. (2009) tested 20 different agro-industrial residues as solid substrate to produce milk-clotting enzyme with the local isolate of *M. circinelloides*. Among the solid substrates studied, dhal husk provided maximum enzyme activity (7,651 U/g), and the yield was improved by supplementing with sucrose and yeast extract. Among all the physico-chemical parameters tested, the best results were obtained in a medium with a moisture content of 20% at pH 7.0, when inoculated with 30% of spore suspension and incubated at 30 °C for 5 days. The addition of Mg²⁺, Ca²⁺, and Cu²⁺ ions increased protease production, while a reduction was observed when Na²⁺ and Zn²⁺ were added. Cultivation parameters for the *Mucor* spp. aspartic proteinases under SmF and SSF conditions have been summarized in Table 2.

Recovery and purification of *Mucor* spp. aspartic proteinases

Any bio-production process, whether it involves SSF or SmF, consists of two major stages: (a) upstream operations and the cultivation process as required for enzyme biosynthesis and (b) downstream processing as required for the efficient recovery and purification of the product.

Since filamentous fungi are good exporters of proteins and enzymes to the extracellular media, the downstream processing routine in this case does not have to include a cell disruption step. The product can be recovered directly from the fermentation liquor or leached out from the solid substrate cultures. Figure 1 depicts the common sequence of unit operations utilized for the downstreaming of extracellular enzymes produced by either SmF or SSF of filamentous fungi. The final degree of purification is dictated by the application which determines the steps necessary for downstream processing.

A prerequisite for enzyme purification is the ability to recover the product in its active form and in a clarified and concentrated solution, where the mentioned macromolecule of interest is stable enough for further processing. When aspartic proteinase(s) are produced in SmF, separation of the mycelia (biomass) is performed either by centrifugation or filtration, and the subsequent clarified broth is concentrated by tangential-flow (ultra) filtration. However, an SSF-produced enzyme has to be extracted out of the solids (fermented mass), and therefore, an additional operation (“leaching”) is introduced. This is an important unit operation required to prepare a concentrated crude extract by utilizing an appropriate solvent (or “extractant”). Leaching performance is affected by various factors like the pretreatment of the fermented mass, the retention of the solvent in the solids, the nature (diffusivity) of the product, the mixing regime in the extraction vessel, the contact time between phases, the system pH and temperature, and the leaching technique. For example, Fernandez-Lahore et al. (1998) pointed out that the leaching efficiency of a sodium chloride solution was higher than that of a non-ionic detergent solution (Triton X-100; Tween 80) or distilled water alone. Concerning the leaching operation itself, Kumar and Losanse (1987) demonstrated that the counter-current method for solids leaching was more efficient than the percolation method.

Crude extracts or fermentation supernatants may contain more than one type of proteinase, or they may contain interfering enzyme activities and/or undesirable secondary metabolites. Belyauskaite et al. (1980) cultivated *M. renninus* via submerged fermentation and found three different proteinases with similar molecular weights (35 kDa) although only one possessed milk-clotting activity. The three proteinases depicted characteristic pH values with maximum proteolytic activity at 4.25, 3.5, and 2.5, respectively. This is an exceptional finding since in most cases *Mucor* spp. produced only one main milk-clotting enzyme, which is typically found as a main component in culture supernatants or crude extracts. In any case, unit operations based on chromatography and adsorption may be required in order to produce an adequate separation between the product and other “contaminants,”

Table 2 Cultivation parameters for the *Mucor* spp. aspartic proteinases

Production	Strain	Max. activity	Peak of enzyme production (h)	Cultivation parameters	Reference
SmF	<i>M. mucedo</i>	190 U/ml	48	24 °C, 220 rpm	Handel and Fraile (1984)
	<i>M. mucedo</i> DSM 809	130 U/ml	48	24 °C, 220 rpm, pH 4.5	Yegin et al. (2010)
	<i>M. circinelloides</i>	–	96 h	25 °C, 120 rpm, pH 5.2	Andrade et al. (2002)
	<i>M. renninus</i>	–	–	30 °C, 0.85 m ³ /m ³ /min ^a , 200 rpm, pH 5.5	Belyauskaite et al. (1980)
	<i>M. rouxii</i>	0.72 U/ml ^b	20	28 °C	Diaz and Ruiz Herrera (1987)
SSF	<i>M. racemosus</i>	6,500 U/g	–	–	Higashio and Yoshioka (1982)
	<i>M. bacilliformis</i> (M-88)	4,000 U/g	85	24 °C, static, pH 5.5	Fraile et al. (1981)
	<i>M. bacilliformis</i>	7,500 U/g	72	24 °C, static, pH 4.5	Fernandez-Lahore et al. (1997)
	<i>M. varians</i> Pisppek	4,000 U/g	–	–	Bernardinelli et al. (1983)
	<i>M. circinelloides</i> (M-105)	7,100 U/g	96	24 °C, static, pH 4.8	Fernandez-Lahore et al. (1999)

^a Aeration rate (m³/m³/min)

^b Activity has been determined by using hemoglobin as substrate (Schlamowitz and Peterson 1959)

which are not necessarily proteins. Fernandez-Lahore et al. (1998) recovered the milk-clotting enzyme from *M. bacilliformis* from SSF crude extracts with 46% yield by employing ethanol (or acetone) precipitation at optimized pH values (e.g., ranging from 4.0 to 5.0). Areces et al. (1992) further purified such aspartic proteinase by employing ion exchange chromatography. Combination of various methods is a common strategy: Diaz and Ruiz Herrera (1987) purified an aspartic proteinase from *M. rouxii*

submerged cultures by a method which involved salt and acid precipitation, anion exchange chromatography, and size-exclusion chromatography.

Crude extracts usually contain a range of secondary metabolites and inorganic salts. At industrial scale, those can be eliminated or reduced by utilizing membrane operations (ultrafiltration and subsequent diafiltration). These combined operations are the equivalent of dialysis, which is commonly employed at the laboratory scale after protein precipitation with ammonium sulfate. Another way to reduce the content of organic molecules and salts or to exchange the solution chemistry is size-exclusion chromatography; this is normally regarded as a “polishing” step. In some cases, the presence of “recalcitrant” small molecular weight organics in a complex biological mixture may induce changes in the chromatographic behavior of a targeted enzyme. Such compounds may alter the solubility and activity of these enzymes and thus negatively affect the performance of chromatographic matrices and ultrafiltration membranes by irreversible fouling. Therefore, it is advisable to implement a strategy specifically designed to sequester recalcitrant organics.

Fernandez-Lahore et al. (1998) designed a process aiming for the efficient and streamlined recovery of bioproducts from SSF. The aspartic proteinase from *M. bacilliformis* was utilized as a case example. The final optimized procedure consisted in the following steps: (a) leaching of the fermented mass with 1% sodium chloride solution, (b) concomitant removal of recalcitrant organics by adsorption onto low-porosity mixed-mode beaded resins, (c) concentration and diafiltration via synthetic membranes, (d) capture onto anion exchangers in finite bath. An overall purification factor of 15.5 and total enzyme yield of 46% were achieved, fitting very well with

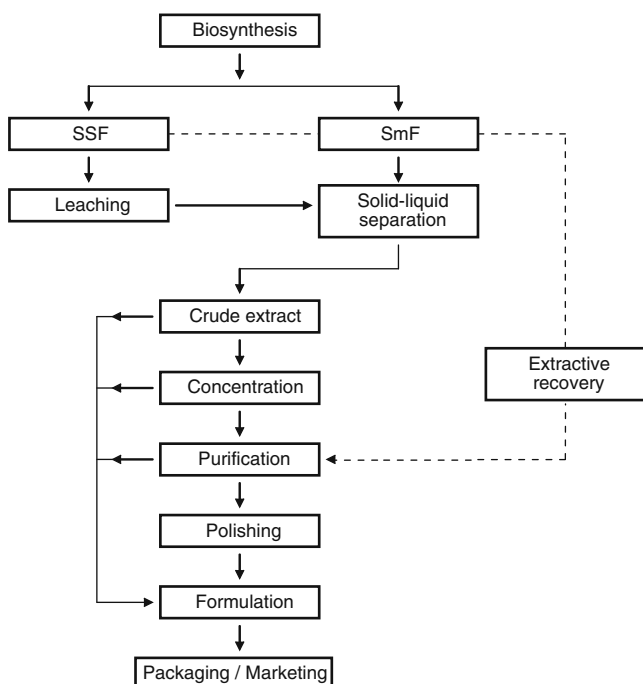


Fig. 1 An integrated sequence of operations for the downstream processing of extracellular aspartic proteinase produced by SSF and SmF systems

the standard procedures found in the literature. A similar strategy was also applied in the recovery and purification of the aspartic proteinase from *M. circinelloides* where a final yield of 76.2% and specific activity (purified enzyme) of 1,280 U/mg was obtained (Fernandez-Lahore et al. 1999). Table 3 summarizes reports on the purification strategies of aspartic proteinases from Mucorales.

The cost associated with product downstreaming and a concomitant increase in global product yields can be obtained by “integrating” recovery and purification unit operations. This concept can be materialized by replacing several traditional unit operations by a novel one which is able to deliver a similar performance. For example, the extractive recovery of enzymes in aqueous two-phase systems (Hustedt et al. 1985) can serve for the simultaneous clarification, concentration, and fractionation of a complex extract. Fernandez-Lahore et al. (1995) reported the extractive recovery and purification of *M. bacilliformis* aspartic proteinase in polymer/polymer and polymer/salt aqueous systems. Enzyme partition behavior was investigated in such systems as a function of polymer type and molecular weight, phase-forming salt type, system pH, and sodium chloride concentration. Suitable conditions for enzyme purification were found by setting a two-step extraction/back-extraction scheme.

An enzyme solution with the appropriate level of purity has to be finally formulated, so as to guarantee product stability, potency, and standardization during final application. Formulation in the liquid and concentrated form usually considers the addition of salts, like sodium chloride, adjustment of pH, and incorporation of various preservatives (citrate, phosphate, ammonium, alkaline metal ions, glycerol, or sorbitol). Powdered enzyme products are typically produced by spray drying in the presence of inert components (maltodextrin, polyethylene glycol, gum arabic, hydrolyzed starches, emulsifying starches, etc.). This technique can be detrimental to the activity of proteinases which are heat labile. Vacuum drying and freeze drying are available alternatives, although processing cost and enzyme

stability could limit the utilization of such techniques at larger scales. Summarizing, formulation is not a trivial step and requires a proper optimization particularly with labile enzymes.

Comparative structure and function of *Mucor* spp. aspartic proteinases

Work describing the structural properties of aspartic proteinases derived from *Mucor* spp. is scarce. Fernandez-Lahore et al. (1999) showed that the aspartic proteinase produced by solid-state cultures of *M. circinelloides* (M-105) has a molecular weight of 33 kDa, as judged by SDS-PAGE analysis and size-exclusion chromatographic experiments. A *pI* value of 4.21 was obtained by the same authors. The optimum pH for proteolytic activity towards dimethylcasein was within the range of 3.0 to 3.5. Regarding thermostability, this proteinase retained 26% of its proteolytic activity at 50 °C and 13% of its proteolytic activity at 60 °C after a 30-min incubation period, at pH 5.0. The enzyme from the M-105 strain was fully inhibited by pepstatin A, and no effect was observed when phenylmethylsulfonyl fluoride, *p*-chloromercuriphenylsulfonate, or ethylenediaminetetraacetic acid was used. N-terminal amino acid sequence was also determined. Figure 2 shows a comparison of N-terminal sequence of M-105 strain with those from other fungal enzymes and bovine chymosin. It can be observed that Tyr14 and Gly21 are conserved in all the proteinases compared, including the one from M-105. But it has to be pointed out that neither Try14 nor Gly21 is structurally related to substrate-binding-specific subsite. Gly may have an important structural role since 11 Gly residues are present among the 34 invariant residues observed. Pro23 and hydrophobic residues, e.g., Val18 and Val20, are also well conserved when comparing primary sequences between proteinases.

Machalinski et al. (2006) reported the structural aspects of aspartic proteinases obtained from a mesophilic *M.*

Table 3 Strategies for purification of aspartic proteinases from Mucorales

Purification strategy	Microorganism	Reference
Affinity chromatography, ultrafiltration, size exclusion	<i>Mucor renninus</i>	Belyauskaite et al. (1980)
Salt and acid precipitation, size exclusion, anion exchange chromatography	<i>Mucor rouxii</i>	Diaz and Ruiz Herrera (1987)
Dialysis, anion exchange chromatography	<i>Mucor bacilliformis</i>	Areces et al. (1992)
Two-phase partition	<i>Mucor bacilliformis</i>	Fernandez-Lahore et al. (1995)
Dialysis, anion exchange chromatography	<i>Mucor bacilliformis</i>	Venera et al. (1997)
Diafiltration, anion exchange chromatography	<i>Mucor bacilliformis</i>	Fernandez-Lahore et al. (1998)
Ultrafiltration, anion exchange chromatography, size exclusion chromatography	<i>Mucor circinelloides</i>	Fernandez-Lahore et al. (1999)
Filtration, ethanol precipitation	<i>Rhizomucor miehei</i>	Thakur et al. (1993)
Precipitation, dialysis, anion exchange chromatography, size exclusion chromatography	<i>Rhizopus oryzae</i>	Kumar et al. (2005)

	1	10	20
<i>E. parasitica</i>	STGSATTTPIDSLDDAYITPVQIGTP		
<i>P. janthinellum</i>	AASGVATNTPTA-NDEEYITPVTIG--		
<i>R. chinensis</i>	AGVGTVPMTDYG-NDIEYYQVVTIGTP		
<i>R. niveus</i>	ASGSVPMVDYE-NDVEYYGEVTVGTP		
<i>M. pusillus</i>	AEGDGSVDTPGLYDF--DLEEYAI PVSIGTP		
<i>M. miehei</i>	AAADGSVDTPGYDF--DLEEYAI PVSIGTP		
Calf chymosin	GEVASVPLTNY--LDSQYFGKIYLGTP		
<i>M. bacilliformis</i>	GTGTVPVTDDG--NDIEYYGTVKVGTP		
M. 105	GTGTVPVTDDG--NLNEYXTVTVGXTP		

Fig. 2 Alignment of the N-terminal sequences of several fungal aspartic proteinases and calf chymosin (Fernandez-Lahore et al. 1999)

bacilliformis strain. The molecular weight of the proteinase was close to 32 kDa. The protein was monomeric, having 323 amino acid residues and four cysteine residues involved in two disulfide bridges. It is known that other members of the aspartic proteinase family have one to three disulfide bridges located at positions 251–286. The *M. bacilliformis* proteinase was reported as non-glycosylated. Although the importance of glycosylation has not been fully established for the aspartic proteinase family, it could stabilize protein conformation, thus leading to higher thermostability. In the cheese industry a low thermostability is a particularly desired rennin property so as to avoid residual proteolysis during maturation. Many commercial microbial rennet preparations are based on the milk-clotting enzymes produced by *R. miehei*. These proteinases possess higher thermostability and have large carbohydrate content (6%, two glycosylation sites). In contrast, proteinases derived from mesophilic strains like the rhizopuspepsin from *Rhizopus niveus*, the pencillopepsin from *Penicillium janthinellum*, and the endothiasepsin from *E. parasitica* are not glycosylated. Sequence alignment of calf chymosin with *M. bacilliformis* proteinase revealed a 33% homology, which is higher than that of proteinases from *R. miehei* (25%) and *E. parasitica* (26%). The *R. miehei* rennet is currently used in cheese manufacture, thus indicating that the *M. bacilliformis* proteinase could be a useful substitute with better technological properties. Figure 3 shows the predicted 3D ribbon-

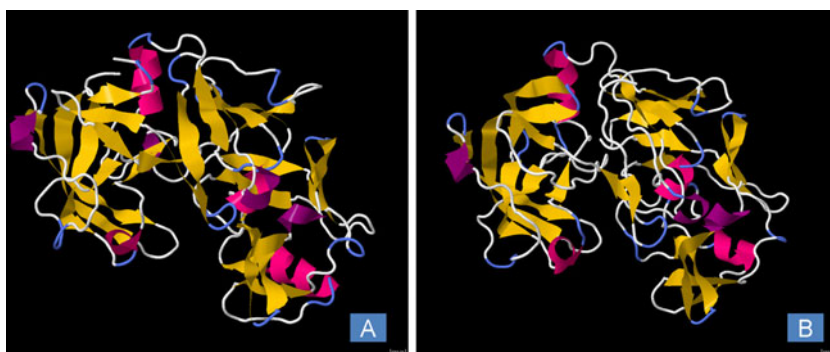
type computational representation models for *M. circinelloides* and *M. bacilliformis* (online homology modeling with Geno3D was used to create structural models).

Venera et al. (1997) performed a detailed biochemical and kinetic study on the milk-clotting proteinase from *M. bacilliformis*. An extinction coefficient, $\epsilon_{278 \text{ cm}} = 1.61 \text{ ml mg}^{-1} \text{ cm}^{-1}$, a molecular mass of 35.4 kDa, and pI of 5.2 were determined for the protein under study. The optimum temperature for proteolytic activity was 45 °C. This behavior resembled bovine chymosin which has an optimum temperature of 47 °C under the same experimental conditions. Contrarily, fungal proteinases from thermophilic strains, such as those from *R. miehei* and *R. pusillus*, which are extensively used in the cheese industry, have activity optima at 60 °C and 55 °C, respectively. In the same study, optimum pH for peptide cleavage was determined as 3.0 and 3.5 for hemoglobin and casein, respectively. Kinetic parameters were also determined for *M. bacilliformis* aspartic proteinases as well as its susceptibility to inhibition by pepstatin A. On the basis of the ratio of catalytic to dissociation constants ($k_{\text{cat}}/K_{\text{m}}$) as measured on the cleavage of a peptide substrate, two subgroups of aspartic proteinases were defined: (a) a first group having $k_{\text{cat}}/K_{\text{m}}$ ratio values higher than $1,000 \text{ mM}^{-1} \text{ s}^{-1}$, e.g., bovine and porcine pepsins and (b) a second group presenting much lower $k_{\text{cat}}/K_{\text{m}}$ ratio, e.g., *Rhizopus* proteinase and bovine chymosin. Catalytic efficiency of *M. bacilliformis* proteinase was found within the later subgroup since the $k_{\text{cat}}/K_{\text{m}}$ values obtained were in the $214 \text{ to } 259 \text{ mM}^{-1} \text{ s}^{-1}$ range, depending on the approach selected to perform the calculations. Thermodynamic and kinetic studies on other *Mucor* spp. aspartic proteinases are, at the best of our knowledge, not available in the literature.

***Mucor* spp. aspartic proteinases in cheese manufacturing and beyond**

Aspartic proteinases can be utilized for different technological purposes. These include milk clotting during cheese

Fig. 3 The predicted 3D ribbon-type computational representation models for *M. circinelloides* (a) and *M. bacilliformis* (b)



manufacturing (Claverie-Martin and Vega-Hernandez 2007), production of seasonings products (Kanlayakrit and Maweng 2006), degradation of turbidity complex resulting from protein in fruit juices and alcoholic liquors (Sumantha et al. 2006), and production of “renneted casein” and “casein hydrolysates” which are used as fat emulsifier and texture modifier agent in food and pharmaceutical formulations (Phelan et al. 2009) and also for increased fiber hydrolysis from feedstock available from agricultural processing operations (Abbas and Bao 2009), meat tenderization (Ashie et al. 2002), and peptide synthesis (Kumar and Bhalla 2005; Filippova and Lysogorskaia 2003). However, there is scarce information available on the application of aspartic proteinases other than in the cheese-making process that has been the preferred area of application until now.

Cheese production using a milk-clotting enzyme or “rennet” is the oldest attempt to extend the utilization of milk. Traditionally, bovine chymosin obtained from the abomasums of unweaned calves has been employed. The bovine “rennet” obtained from adult animals is, in contrast, rich in pepsin and has poorer technological properties. Milk-clotting enzymes are also commercially available from other mammalian species, e.g., lamb, goat, pig (Nielsen and Foltmann 1995), or from microbial sources. All of them have found well-defined applications in the dairy industry. Among the microbial milk-clotting enzymes, the ones produced by *R. miehei* and *R. pusillus* have gained wide industrial acceptance as substitutes for the bovine chymosin (Claverie-Martin and Vega-Hernandez 2007). However, it is well known that fungal “rennet” preparations may present a degree of tertiary (or “residual”) proteolytic activity resulting in the production of bitter peptides during cheese ripening. Bovine chymosin, which remains the industrial gold standard, produces a very specific cleavage of the Phe¹⁰⁵-Met¹⁰⁶ bond of κ -casein, therefore destabilizing the casein micelle structure (Turhan and Mutlu 1998). Therefore, cleavage specificity as opposed to extended proteolysis defines a good milk coagulant.

Enzymatic milk coagulation is a two-phase process; any variation in the chemical environment can affect the two stages of reaction separately. In the first phase, the enzyme hydrolyzes the κ -casein molecule, splitting the protein into two fragments: the (hydrophobic) para- κ -casein and the (hydrophilic) macropeptide. The latter is able to diffuse into the milk whey. The second phase consists of the coagulation of the casein micelles that have been destabilized by proteolytic attack (Claverie-Martin and Vega-Hernandez 2007). This phase is sensitive to temperature, medium pH, and Ca²⁺ ion concentration. Figure 4 shows the mechanism of milk coagulation by bovine chymosin.

Any proteolysis caused by milk protein fractions other than the specific action on the para- κ -casein is considered

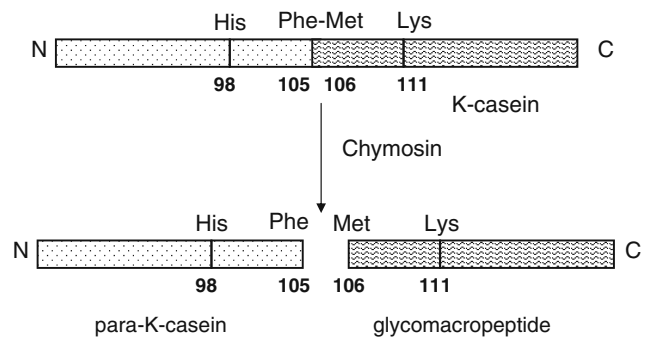


Fig. 4 Mechanism of milk coagulation by chymosin

undesirable. This phenomenon is very significant since it can be considered responsible for poor cheese quality, decreased curd yield, and economic loss. A heat-labile enzyme would be eliminated after the intended purpose of milk coagulation during subsequent manufacturing steps that may include heating. *Mucor* spp. aspartic proteinases presenting a high milk-clotting to tertiary proteolysis ratio and notorious thermosensitivity could find widespread application in cheese manufacturing. Recently, Sathya et al. (2009) successfully applied aspartic proteinase from *M. circinelloides* in the manufacture of cheddar cheese and compared it with the commercial rennet. Aspartic proteinase obtained from *M. circinelloides* was found to be nearly equal in yield to that made with the commercial rennet, in that 1 l of cows’ milk produced 163 g of cheese using the purified milk-clotting enzyme compared with 172 g produced by the commercial rennet. Tubesha and Al-Delaimy (2003) reported that the production of cheese from local isolate of *Mucor* J20 strains was 160 g/l of milk sample. It has been pointed out by Fraile et al. (1981) and Fernandez-Lahore et al (1997) that like calf rennet aspartic proteinase from *M. bacilliformis* and *M. circinelloides* loses activity with heat treatment. The milk-clotting enzyme from thermophilic fungi like *R. pusillus* kept 71% of its original activity when heated for 10 min at 55 °C.

Aspartic proteinases from *Mucor* spp. have the potential to find diverse applications in the food and beverages industry and beyond, including, for example, the (bio) chemical and pharmaceutical industries. Concerning applications in food, recent progress has targeted the dairy and cheese manufacturing industries. This has been the direct consequence of understanding some of the advantageous properties of *Mucor* spp. proteinases, particularly thermosensitivity and limited proteolytic activity. But much effort has to go into the testing of such enzymes for many other potential niche applications.

A large number of *Mucor* spp. strain are readily available, but not exploited yet, for aspartic proteinase(s) production. Moreover, these strains are amenable to industrial cultivation by submerged or solid substrate

fermentation systems. Downstream techniques are also known for the efficient recovery and purification of these enzymes. The latter may even include the utilization of affinity “tailor-made” peptides or peptide analogs as proteinase ligands. The application of classical mutation and screening technologies and genome shuffling procedures coupled to media design and efficient fermentation procedures can deliver processes depicting a high level of enzyme production. Since no genetic engineering procedures are utilized in this case, no special food labeling is required. Therefore, consumer acceptance of such products is favored.

Nowadays, it is also possible to clone the genes encoding aspartic proteinases and express them by employing host microorganisms that are well adapted to industrial-scale fermentation. This approach would lead to the mass production of aspartic proteinases with unique technological properties. For example, protein engineering tools that eventually will permit further improvement (or adaptation) of enzyme properties are available. This will open a completely new era in the understanding and application of *Mucor* spp. aspartic proteinases.

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