

Full Length Research Paper

Production of extracellular aspartic protease in submerged fermentation with *Mucor mucedo* DSM 809

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Accepted 21 June, 2010

Fungal milk-clotting enzymes have gained value as bovine Chymosin substitutes in the cheese industry. In this work, the effects of culture conditions on the production of extracellular milk clotting enzymes from *Mucor mucedo* DSM 809 in submerged fermentation were studied. 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, 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 SU per ml fermentation broth. The inoculum type and size has also affected biomass production and the biosynthesis of the enzyme. The preferred method was the inoculation of the culture media with spores at a total load of 6×10^5 spores per flask.

Key words: Milk clotting enzyme, Aspartic protease, *Mucor mucedo*, Sub-merged fermentation.

INTRODUCTION

Aspartic proteases are a group of proteolytic enzymes produced by many microorganisms. A significant property of aspartic proteases is the ability to coagulate milk, as is evidenced by their widespread application in the dairy industry to coagulate casein during the manufacturing of cheese. Calf rennet has been traditionally utilized for milk coagulation and curd formation. Milk clotting by calf rennet occurs essentially by cleaving the Phe₁₀₅-Met₁₀₆ bond of *k*-casein, resulting in the release of a hydrophilic (macro) glycopeptide, which passes into the whey. Para-*k*-casein becomes positively charged at neutral pH and causes decrease of the repulsive forces between casein micelles thereby causing aggregation and curd formation (Vishwanatha et al., 2009).

The limited supply of natural calf rennet has simulated the search for alternative sources of milk-clotting enzymes of microbial origin and the production of recombinant bovine chymosin. Studies were mainly focused on fungal

aspartic proteases although a few reports on bacterial enzymes can be found (Dutt et al., 2008). Filamentous fungi are the major producers of microbial aspartic proteases. Fungal substitutes for animal proteases proposed include those from *Rhizomucor pusillus* (Ismail et al., 1984), *Rhizomucor miehei* (Preetha and Boopathy, 1994; Escobar and Barnett, 1993), *Mucor bacilliformis* (Arecas et al., 1992; Venera et al., 1997; Fernández-Lahore et al., 1997; Machalinski et al., 2006), *Mucor circinelloides* (Fernández-Lahore et al., 1999), *Rhizopus oryzae* (Kumar et al., 2005), *Aspergillus oryzae* (Vishwanatha et al., 2009), *Endothia parasitica* (Sardinas, 1968), *Penicillium oxalicum* (Hashem, 1999), *Amylomyces rouxii* (Yu and Chou, 2005) and *Fusarium subglutinans* (Ghareib et al., 2001). Among the several microorganisms studied, *Rhizomucor miehei* and *Rhizomucor pusillus* have gained industrial acceptance as producers of milk-clotting enzymes.

Commercial rennet preparations usually contain tertiary (or unspecific) proteolytic activities, which may further degrade curd proteins, leading to its dissolution and to the production of bitter peptides. Another striking feature that hinders the use of microbial rennets is their high thermal stability which allows them to extend their action on milk proteins after coagulation. Although after cheese making

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only 0 to 15% of the rennet activity added to milk is retained in curd (Sousa et al., 2001), the mentioned characteristics of commercial fungal rennets may cause extensive proteolysis resulting in low cheese yields and poor product quality. Particularly, soft and semi-hard cheese varieties are affected by extensive proteolytic activities (Hynes et al., 2001). Moreover, excessive proteolysis may degrade proteins of economic value that are present in the cheese whey.

A possible solution to alleviate the technical challenges described before is the identification of milk-clotting enzymes with enhanced specificity and reduced thermo tolerance. This may allow for limited proteolytic action and subsequent rapid destruction of the protease during further manufacturing operations e.g. heating. Fernández-Lahore et al. (1999) proved that aspartic proteases produced from certain mesophilic *Mucor sp. strain* (*M. bacilliformis* and *M. circinelloides*) have, like bovine chymosin, less heat stability than those from thermophilic fungi. However, the production of the aspartic proteinases from such microorganisms was only possible *via* solid state fermentation. On the other hand, *M. mucedo* has been screened as a mesophilic fungi possessing milk clotting activity (Fraile et al., 1978) and some of the factors affecting the production has been already reported by the use of local isolates (Handel and Fraile, 1984; Mashaly et al., 1981).

The aim of the present study was to investigate the growth conditions of *Mucor mucedo* DSM 809 in submerged fermentation to explore the production of milk-clotting enzymes and to obtain a better understanding on the biology of that microorganism during cultivation in shake flasks. Since other mesophilic *Mucor sp.* strains have been reported as producers of aspartic proteinases only under solid state fermentation conditions, *M. mucedo* can be a promising microorganism for milk-clotting enzyme production in submerged fermentation. The later is widespread in industry as a process that can be readily scaled-up for enzyme production.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The microorganism used for the production of the milk-clotting enzyme was *Mucor mucedo* DSM 809, obtained from the German Collection of Microorganisms and Cell Cultures-DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). The strain was maintained by subculturing on potato-dextrose agar (PDA) slants at 24°C. Spores were harvested after 5 days of surface cultivation by suspension in sterilized distilled water. Inoculum size was adjusted by manual spore counting in a Neubauer chamber.

Growth Medium for submerged fermentation

To study effect of pH, carbon source, nitrogen source, shaking speed, and inoculum size experiments were carried out in duplicate Erlenmeyer flasks (250 ml) containing 30 ml of sterilized synthetic growth. The medium was inoculated with a spore suspension at the desired spore concentration.

The cultivation media consisted of a carbon source, a nitrogen source, and a mineral solution which were sterilized separately. The production media consisted in 1% (w/v) of the carbon source and 0.5 % (w/v) of the nitrogen source. Glucose, fructose, lactose and maltodextrin were tested as a carbon source while tryptone, casein and skim milk powder were tested as a nitrogen source. Casein concentration was further optimized. Carbon and nitrogen sources were autoclaved for 20 min at 120°C. The final concentration of each mineral in media is as follows (in g/L): $\text{SO}_4\text{Mg}\cdot 7\text{H}_2\text{O}$ 0.49; $\text{SO}_4\text{Zn}\cdot 7\text{H}_2\text{O}$ 0.0018; $\text{SO}_4\text{Mn}\cdot \text{H}_2\text{O}$ 0.0003; $\text{SO}_4\text{Cu}\cdot 5\text{H}_2\text{O}$ 0.0004; $\text{SO}_4\text{Fe}\cdot 7\text{H}_2\text{O}$ 0.001; and KH_2PO_4 4.49. The mineral solution was prepared as a concentrate, adjusted to the final pH and sterilized by autoclaving (20 min at 121°C). 0.6 ml of this solution was added to 29.4 ml of the C/N production media in each of the flasks. Biomass was harvested by filtration and the filtrate was assayed for enzyme activity and other biochemical parameters.

Analytical procedures

Assay for milk clotting activity

Milk clotting activity was determined according to the method of Arima et al. (1970), which is based on the visual evaluation of the appearance of the first clotting flakes, and expressed in terms of Soxhlet units (SU). One Soxhlet unit is defined as the amount of enzyme that clots 1 ml of substrate in 40 min at 35°C. In order to perform the assay, 0.1 ml of the sample was added to a glass test tube containing 1 ml of reconstituted skim milk solution (10 g skim-milk powder dissolved in 100 ml of 0.01 M CaCl_2 solution) pre-incubated at 35°C for 10 min. The mixture was mixed well and the clotting time T (s) was measured with a chronometer. The clotting activity was calculated using the following formula:

$$\text{SU} = (2400 \times 1 \times \text{D}) / 0.1 \times \text{T}$$

D: dilution of sample material
T: clotting time (s)

Assay for protease activity

Protease activity was determined by the azocasein digestion method, essentially according Samal et al. (1990). To a final volume of 500µl; 20 µl of azocasein substrate (5% w/v azocasein solution in 200mM Tris-HCl buffer, pH 7.5), 20 µl enzyme sample and 460 µl of 50 mM Tris HCl buffer, pH 7.5, were added. Following the incubation for 30 min at 37°C, 500 µl of TCA (10% w/v) was added and the samples were kept on ice 15 min. After centrifugation 7000 rpm for 10 min, 800 µl of supernatant was added to a tube containing 200 µl of NaOH (1.8 N). Absorbance was measured at 420 nm. The blank for each sample was prepared separately in the same manner except 500 µl of TCA was added before the addition of enzyme sample. One unit of protease activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.003 under the above assay condition.

Protein determination

Protein was determined according to the Bradford procedure with bovine serum albumin as the standard (Bradford, 1976).

Carbohydrate determination

Total carbohydrate was determined employing the phenol-sulphuric-acid procedure as *per* Dubois et al. (1956). Glucose was utilized as

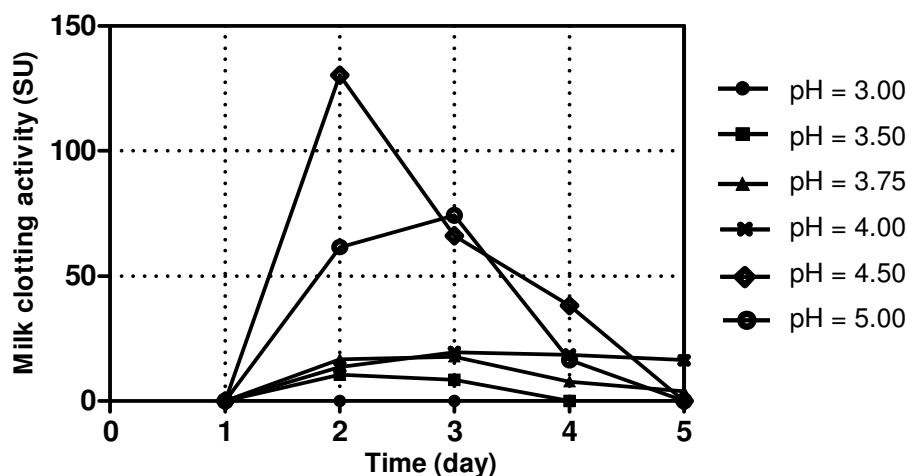


Figure 1. Effect of initial pH on milk clotting activity for 5 days fermentation period (Media: 1% glucose + 0.5% casein + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; inoculum concentration: 2×10^4 spore/ml; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

standard.

Biomass determination

For biomass determination, samples of the culture (30 ml) were clarified by filtration through a Whatman filter paper (\varnothing 90 mm). The separated solids (biomass) were dried until constant weight at 80 °C (Silveira et al., 2005).

RESULT AND DISCUSSION

Effect of initial pH

The pH of the medium is a very important but often neglected environmental factor. It can profoundly affect any biological activity being studied (Papagianni, 2004). In this study, pH was found to be a prevailing factor affecting milk-clotting enzyme production. The effect of initial pH of the growth medium on the enzyme activity during the course of a 5 days fermentation period is shown in Figure 1. The optimum initial pH value for maximum enzyme activity was found to be 4.50 (130 SU). No milk-clotting activity was detectable at pH 3.0 under similar cultivation conditions. It is evident that pH is a critical parameter influencing maximum production levels and enzyme biosynthesis kinetics. Mashaly et al. (1981) cultivated *M. mucedo* and found that the ratio milk clotting activity / proteolytic potency was higher when the initial pH value was adjusted to 3.75 while maximum milk-clotting enzyme production by *R. pusillus* (Ismail et al., 1984), *Amylomyces rouxii* (Yu and Chou, 2005), *Fusarium subglutinans* (Ghareib et al., 2001), and *Bacillus licheniformis* (D'Souza and Pereira, 1982) were reported to be maximum with an initial pH of 3.7, 7.0,

6.0 and 7.0 respectively. The optimal initial pH of the medium for milk-clotting enzyme production obviously may vary depending on the culture medium and microbial organism under the study. Summarizing, the initial pH of the cultivation media is a parameter impacting on both maximum enzyme production levels and on the properties of the crude extract.

Effect of inoculum size

Three inoculums size values were tested: 2×10^3 , 2×10^4 , 2×10^5 spores per ml of medium. The variation on inoculum size affected the biosynthesis of enzyme. A large inoculum decreased the level of enzyme obtained as observed by Fernández-Lahore et al. (1997). The maximum activity was obtained with a concentration of 2×10^4 spores/ml, as is shown in Figure 2.

Effect of shaking speed

The production of the milk-clotting enzyme by *M. mucedo* DSM 809 as a function of shaking speed is shown in Figure 3. Among the shaking speed examined (220, 270, and 350 rpm), it was observed that milk-clotting enzyme production was highest at 220 rpm. Enzyme production decreased as the shaking speed increased, probably due to excessive shear stress which could damage biomass integrity. In contrast with Escobar and Barnett (1993), who claimed that the enzyme production was directly proportional to the shaking speed for *R. miehei*, the biosynthesis of the milk-clotting enzyme by *M. mucedo* DSM 809 decreased when the shaking speed increased above

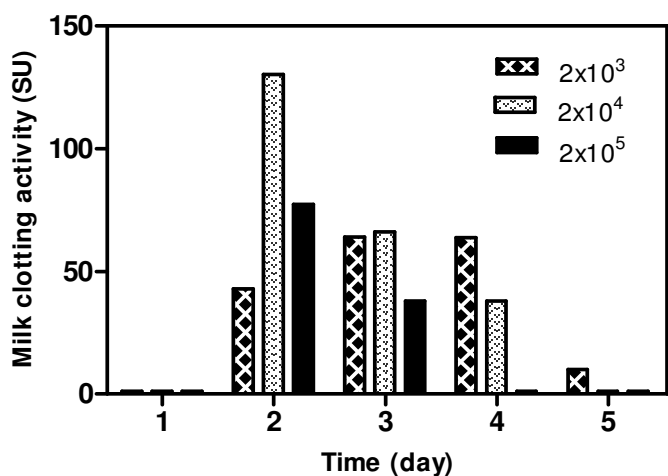


Figure 2. Effect of inoculum concentration on milk clotting activity for 5 days fermentation period (Media: 1% glucose + 0.5% casein + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; initial pH of the medium 4.50; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

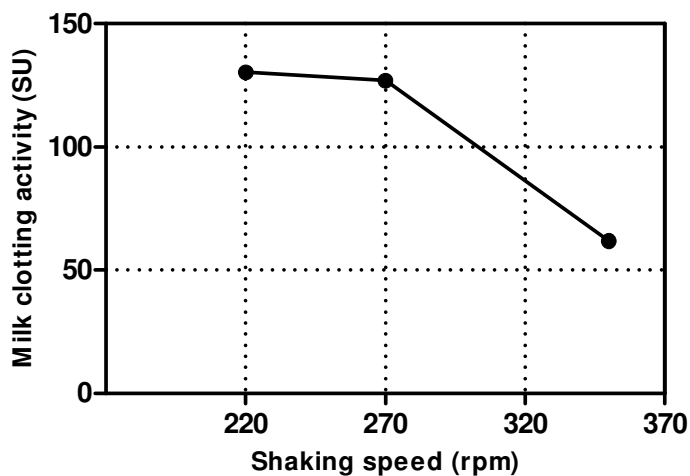


Figure 3. Effect of shaking speed on milk clotting activity at second day of fermentation (Media: 1% glucose + 0.5% casein + 2% mineral solution; cultivation temperature: 24°C; initial pH of the medium 4.50; inoculum concentration: 2×10^4 spore/ml).

certain limits. The findings of the present study are similar to the observations made by Yu and Chou (2005) and Hashem (1999) who reported less milk-clotting activity with increased shaking speeds for *Amylomyces rouxii* and *Penicillium oxalicum*, respectively.

Effect of carbon source

To determine the most favorable source of carbon for

enzyme production, either glucose, fructose, lactose, or maltodextrin were individually employed as a sole carbon source in the production medium. Figure 4 shows the activity levels obtained for each type of carbon source in the course of a 5 days fermentation period. Carbon sources stimulate rennet production depending on the metabolism of the microorganism under the study, as is the case of glucose for *R. miehei* fermentation (Silveira et al., 2005). Similarly, in the present study maximum enzyme activity was observed when glucose was used as a sole carbon source. On the contrary, no activity was obtained when lactose was employed. Channe and Shewale (1998) found that starch was a better carbon source for *A. niger* MC4 while Dutt et al. (2008) obtained maximum enzyme activity when fructose was used as a carbon source for *Bacillus subtilis*. Hashem (1999) found sucrose to be the most favorable carbon source for *Penicillium oxalicum*.

Effect of nitrogen source

Figure 5 depicts the influence of the nitrogen source on the biosynthesis of the milk-clotting enzyme by *M. mucedo* DSM 809. Among the nitrogen sources tested e.g. casein, tryptone, and skim-milk powder, the mentioned strain produced maximum enzyme levels in the presence of casein. On the other hand, the milk-clotting activity was the lowest when skim-milk powder was the sole organic nitrogen source. Silveira et al. (2005) showed that casein played an important role in rennin production under both solid state fermentation and submerged fermentation conditions in the case of *R. miehei*. According to Channe and Shewale (1998), casein triggered higher activity than tryptone and skim milk powder, when they were added separately to a fermentation media; the formation of milk clotting enzyme was very low when skim-milk powder was the sole source of nitrogen for *A. niger* MC4. Preetha and Boopathy (1994) observed that addition of skim-milk powder to the cultivation media did not improve the ratio of milk clotting activity to proteolytic activity during fermentation of *R. miehei*. Dutt et al. (2008) also tested several nitrogen sources for milk-clotting enzyme production by *Bacillus subtilis*. Their data indicate that addition of tryptone provided higher activity than the utilization of casein. From these studies it is clear that the optimal nitrogen sources vary considerably when comparing bacterial vs. fungal producers of milk-clotting enzymes.

Effect of casein concentration

As mentioned before casein proved to be an excellent substrate for the production of the milk-clotting enzyme from *M. mucedo* DSM 809. For this reason, casein levels were further optimized. Figure 6 shows the effect of casein concentration (0.5, 1.0, 1.5 and 2.0% w/v) on the enzyme

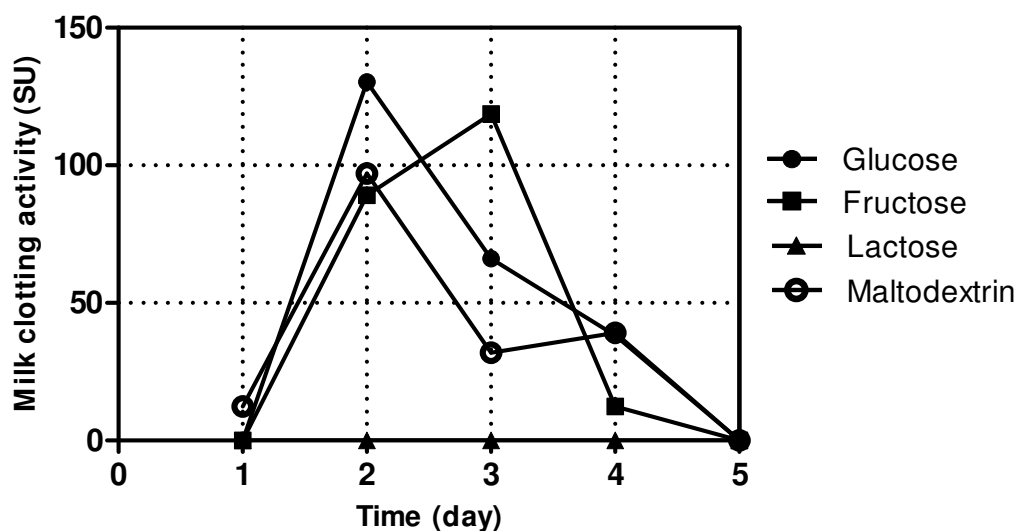


Figure 4. Effect of carbon source on milk clotting activity for 5 days fermentation period (Media: 1% carbon source + 0.5% casein + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; initial pH of the medium 4.50; inoculum concentration: 2×10^4 spore/ml; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

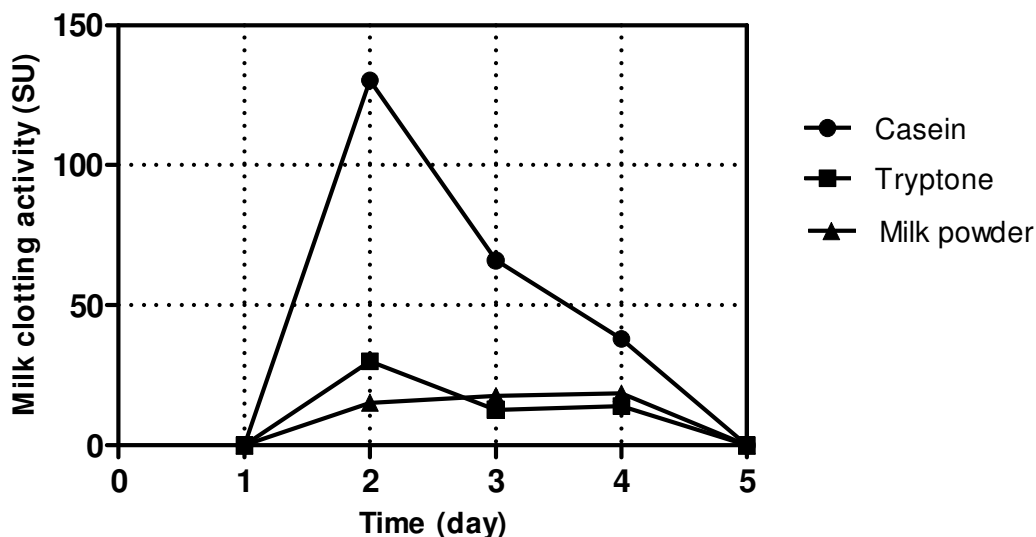


Figure 5. Effect of nitrogen source on milk clotting activity for 5 days fermentation period (Media: 1% glucose + 0.5% nitrogen source + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; initial pH of the medium 4.50; inoculum concentration: 2×10^4 spore/ml; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

production. The maximum milk-clotting activity was obtained when the casein concentration was set at 0.5% (w/v). Under such conditions, a peak of production was clearly observed after 48 h of cultivation. Similarly, Handel and Fraile (1984) also observed that *M. mucedo* produces maximum levels of a milk-clotting enzyme when the casein concentration in the cultivation media was set within the range 0.5 - 0.7% (w/v).

Culture profile and enzyme production

After modifying several nutritional and operational factors effecting the enzyme production during the course of the present study, a final cultivation profile was obtained for *M. mucedo* DSM 809. Table 1 shows the evolution of various cultivation parameters alongside the time of fermentation. It can be observed that the pH increased from 4.5 (at initial

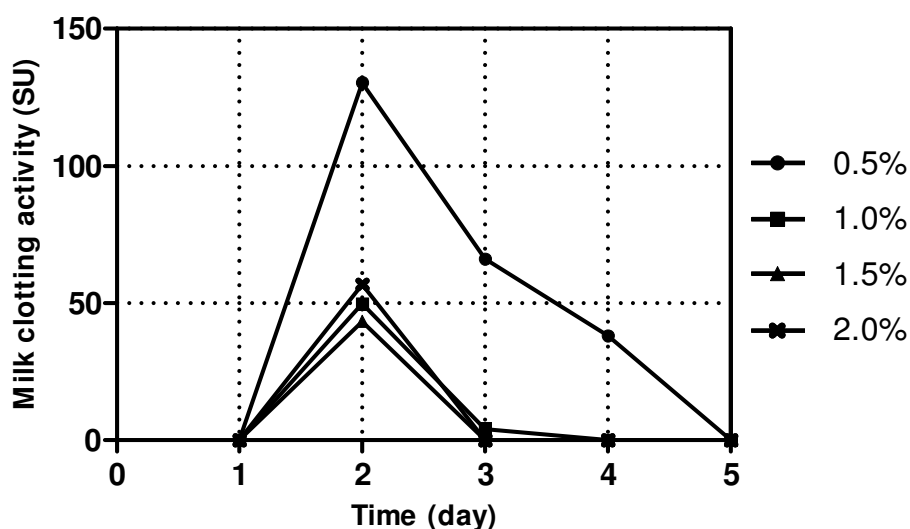


Figure 6. Effect of casein concentration on milk clotting activity for 5 days fermentation period (Media: 1% glucose + casein (0.5, 1.0, 1.5 and 2.0%) + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; initial pH of the medium 4.50, inoculum concentration: 2×10^4 spore/ml; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

Table 1. Final cultivation profile for *M. mucedo* DSM 809

Time (day)	pH	Milk clotting activity (SU)	Protease activity (PU)	MCA/PU ^a	Biomass (g/l)	Total CHO (g/l)	Total protein (µg/ml)
1	3.82±0.04	0	4.8±0.7	ND ^b	3.1±0.2	7.4±0.3	34.22±1.68
2	3.89±0.01	130.7±1.2	7.9±0.5	16.5	6.3±0.8	0.3±0.1	36.90±0.48
3	4.38±0.20	66.1±0.3	7.6±0.9	8.7	6.5±0.4	0.4±0.1	31.82±0.04
4	6.41±0.02	38.1±0.1	1.1±0.1	34.6	6.1±0.3	0.4±0.1	34.95±0.93
5	6.86±0.02	0	1.8±0.2	ND ^b	5.4±0.1	0.3±0.3	39.91±0.01

MCA/PU^a = Milk clotting activity/Protease activity

ND^b = Not determined

conditions) to 6.86 (after 5 days of cultivation). The maximum level of milk-clotting activity was observed at the second day of fermentation. Biomass levels increased from 3.1 g/l at the first day of cultivation to 6.5 g/l after 2-3 days of fermentation, to decrease afterwards. Biomass development was parallel to enzyme production, as it is expected for a primary product of metabolism. Total protease activity reached a maximum level (7.9 PU) on the second day of the fermentation as it was the case for the milk-clotting activity (130 SU). The total carbohydrate content decreased to negligible amounts at the second day of cultivation since it was consumed for biomass and enzyme synthesis.

Conclusion

The production of the milk-clotting enzyme by *M. mucedo*

DSM 809 has been studied in submerged fermentation and the optimized cultivation profile was reported. The mentioned microbial strain could be a valuable source of an enzyme having a high milk-clotting to tertiary proteolysis ratio and decreased resistance to thermal treatment. *M. mucedo* is unique among other *Mucor* sp. strains in its ability to produce a milk-clotting enzyme in submerged fermentation as opposed to solid substrate cultivation. 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.

ACKNOWLEDGEMENTS

This work was supported by BMBF-ERA-Net, euro TransBio-3, BIO-010103441508 (JUB 5130-50290).

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