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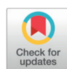

Original Article

Production of Extracellular Rennin-Like Enzyme by a Newly Isolate *Mucor circinelloides* (von Tieghem) and its Application in Camembert Cheese Making

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Abstract

Introduction: Aspartic proteases produced by different non-pathogenic fungi belonging to mucorales, are commonly used as milk-coagulants. The present study aims to optimize conditions for milk-clotting protease production by a new strain of Mucor genus and to assess its ability in soft cheese making.

Materials and Methods: About 20 fungal strains isolated from soil were investigated for their potential to produce milk-clotting proteases for further applications in cheese making. The hyper producer strain *Mucor circinelloides* 2095-2047 was selected for optimization of rennin-like enzyme production under solid-state fermentation (SSF) using the stepwise modifications of the selected parameters. The enzyme produced under the optimal conditions was partially purified and then applied in Camembert cheese making trials compared to the crude extract and commercial rennet.

Results: The maximum milk-curdling activity achieved after optimization (571.43 SU/mL) was obtained using wheat bran (10 g) as the mainly source of carbon containing 1% galactose; moistened with the M-9 solution (pH 6.0) and incubated at 30°C for 96 hours. The enzyme of *M. circinelloides* was partially purified with a high recovery of 105% and 6.23-fold purity after $(NH_4)_2SO_4$ fractionation and dialysis. The physicochemical properties of the three produced cheeses were very close. A low sensory quality of cheese was obtained with the crude extract which was getting better using the pre-purified enzyme. This extract was able to develop a very close or even a similar sensory quality to that obtained by the commercial rennet.

Conclusions: According to findings, it is possible to propose the purified enzyme of *M. circinelloides* as a new alternative for rennet, but further optimization, purification and cheese production tests are required.

Keywords: *Mucor circinelloides*, Proteases, Milk-Clotting Activity, Cheese, Camembert, Solid-State Fermentation

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Introduction

Milk as the rawest material of biological origin is a highly perishable environment that changes rapidly by microbial and enzymatic pathways. It can also subject to various bioconversions that contribute to significantly expand its sensory and nutritional qualities. One derivation of these transformations is cheese. The basic steps of processing milk into cheese for the most varieties are: acidification, coagulation, dehydration, shaping and salting.[1,2] Coagulation of casein, which represents the main protein fraction in milk, to form a coagulum can be realized by addition of milk-clotting enzymes (MCEs), lowering the milk pH to about 4.6 at 30-36°C using lactic acid bacteria or by direct acidification with food-grade acidulants usually gluconic acid-δ-lactone according to the types of cheese.[2,3]

Calf chymosin or rennin (EC 3.4.23.4) is widely considered as the ideal curdling enzyme in cheese manufacture, extracted from the inner mucosa of the fourth stomach of suckling calves as the main constituent (80%-90%), while the second compound pepsin (EC 3.4.23.1) contributes with ~10% of the milk-clotting activity of rennet. The percentage of the two proteinases changes with the ages of the animal which allow to decrease the clotting to proteolytic activity ratio.[2-4]

Chymosin extracted from calf rumen is relatively expensive and has limited supply. Extensive studies have been carried out to search for alternative coagulants (rennet substitutes) from other resources.[2,5] Actually, more than 70% of the global chymosin market is represented by recombinant chymosin[6] produced by genetically modified microorganisms (*Escherichia coli*, *Kluyveromyces lactis* and *Aspergillus niger*).

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These rennets have given excellent results in many cheese varieties but their use is subject to regulation.[3-7]

With the debate developed on the safety of genetically modified products, many researchers are returning to study microbial proteases produced naturally (now called coagulants) by some yeast and fungi, whereas bacterial coagulants are characterized by a high proteolytic activity leading to low quality of cheese.[7,8]

Fungal species are considered as an excellent source of milk coagulants, characterized by their adaptation to culture on solid medium using agro-industrial wastes, which improves yields in enzymes productivity and reduces operating costs.[9,10] Acid proteinases produced by *Cryphonectria parasitica, Rhizomucor pusillus* and *R. miehei* are widely used in industrial cheese production, where no difference between the cheeses produced by these proteolytic enzymes and curdled with rennet have been reported.[2,5,7,11,12]

Several studies have been published in regards to the isolation of microbial strains qualified as milk-coagulants producers, optimization of the cultures conditions and purification of these enzymes, but few of them reached the application in cheese making assay.

The aim of the present study was to evaluate the capacity of a newly isolated strain identified as *Mucor circinelloides* (von Tieghem) to produce an MCE under solid-state fermentation (SSF) as well as optimization of some parameters of the process to maximize the enzyme production. After a partial purification step, Camembert cheese making trials were carried out to evaluate the efficiency or otherwise of the enzymatic extracts produced by the strain as substitutes for commercial rennet.

Materials and Methods

Isolation and Screening of Fungal Isolates for Milk-Clotting Activity

Soil samples (100 g) taken from a depth of 10 to 15 cm from the surface using a sterile spatula from agricultural areas (Bouira and Tipaza, Algeria) were used to search fungal strains producing milk-clotting proteases. Isolation of the fungal strains was performed by cultures on Sabouraud Agar supplemented with Chloramphenicol (0.05 g/L) and Gentamicin (0.02 g/L) to inhibit bacterial growth. A total of 100 µL of each dilution (ranging from 10^{-1} to 10^{-4}) prepared from the stock solution (1 g of soil in 10 mL of sterile normal saline solution) was spread on Petri dishes and then incubated for 120 hours at 28 ± 2°C. All dilutions were prepared in duplicate and plated in triplicate. In order to purify isolates, mycelial fragments from the obtained colonies were transferred to fresh Sabouraud Agar and incubated under the same conditions. The operation was repeated until obtaining pure isolates. The purified strains were stored on Potato Dextrose Agar slants at 4°C.

In order to evaluate the potential of the fungal isolates for MCE production, 5 g of wheat bran were introduced in 250 mL Erlenmeyer flasks supplemented with 4 mL of the mineral solution (SM) (g/L): NH_4NO_3 5, KH_2PO_4 2, $MgSO_4 \cdot 7H_2O$ 1, NaCl 1. After sterilization (121°C, 20 minutes) and cooling, the flasks were inoculated with 1 mL of spore suspension of the isolated strain and incubated at 28 ± 2°C for 96 hours.[13]

Morphological and Molecular Identification

The strain STIP1 characterized by the highest milk-clotting activity was identified according to its phenotypic (macroscopic and microscopic observations that provide a preliminary identification of the strain genus) and genotypic characteristics.

The molecular identification of the strain was carried out at the Belgian Co-Ordinated Collections of Micro-Organisms, Laboratory of Mycology, Catholic University of Louvain-la-Neuve (BCCM-MUL, Belgium) as described by White et al.[14] The DNA extraction was carried out using QIA quick R Genomic Extraction Kit and the polymerase chain reaction (PCR) was performed for ITS1-5.8S rDNA-ITS2 region of ribosomal DNA using the following primers: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The amplification was conducted in DNA thermal cycler (Biometra, Analytik Jena AG, Jena, Germany) at different steps. It consisted of an initial denaturation step at 94°C for 5 minutes and 35 cycles of denaturation step at 94°C for 1 minute followed by annealing step at 56°C for 1 minute and extension step at 72°C for 2 minutes. The final extension step was carried out at 72°C for 10 minutes. At the end of these reactions, the PCR products and their restriction fragments were visualized using 1.5% agarose gel electrophoresis. Moreover, the obtained sequence was aligned with all nucleic sequences deposited in GenBank database using the Basic Local Alignment Search Tool nucleic (BLASTn analysis, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was established with the neighbour joining method using the MEGA 6 Software.

Production of Enzyme under SSF

Since the nature of the solid substrate employed is the most important factor affecting SSF process, three agro-industrial residues available locally with low cost: wheat bran (*Triticum turgidum* ssp. *durum* Desf.), barely bran (*Hordeum vulgare* L.), corncobs (*Zea mays* L.) and their flours were tested for milk-clotting protease production by the selected strain as the mainly sources of carbon. After drying, all the substrates were grinded and sieved to have the same particles size. The fermentation was carried out in 250 mL Erlenmeyer flasks containing 5 g of each substrate, moistened with SM solution to achieve a moisture level of 50.1%. The initial pH of all substrates was adjusted to 6.0 prior to autoclaving. After sterilization at 121°C for 20 minutes, the substrates were inoculated with 1 mL of spore suspension containing 1.75 × 10^8 spores/mL and incubated for 96 hours at 30°C.

After the selection of the best substrate, the second step was to evaluate the effect of the mineral solution to be added to the substrate. For this, Czapek-Dox (g/L: $NaNO_3$ 2.5; KH_2PO_4 1; $MgSO_4 \cdot 7H_2O$ 0.5; KCl 0.5), M-9 (g/L: NaH_2PO_4 12.8; KH_2PO_4 3; NaCl 0.5; NH_4Cl 1; $MgSO_4 \cdot 7H_2O$ 0.5; $CaCl_2 \cdot 2H_2O$ 0.01), M-15 (g/L: NH_4NO_3 3; KH_2PO_4 1; $MgSO_4 \cdot 7H_2O$ 1; $FeSO_4 \cdot 5H_2O$ 0.01) and SM salt solutions[13,15] were tested, compared to the distilled water as the control.

The other studied factors and their employed values were

as follows: amount of the selected substrate (5, 10, 15, 20 and 25 g), addition of simple or complex Co-carbon sources at 1% (w/w) (glucose, fructose, galactose, xylose, sucrose, starch, beans and pods of carob powders), addition of nitrogen sources at 1% (w/w) (urea, ammonium nitrate, ammonium sulfate, yeast extract, casein, meat extract and casein peptone) and fermentation period (24, 48, 72, 96, 120, 144 and 168 hours).

All assays were carried out in triplicate and each result is the mean value of three trials ± SD (Standard Deviation). The confidence level for statistical significance was considered at $P<0.05$.

Enzyme Extraction

The produced MCE and the other metabolites were extracted from the fermented substrate with a precise volume of distilled water corresponding to a ratio of 1:5 (w/v) for 2 hours at 160 rpm[16] on a rotary shaker (MaxQ 4000, Model 4331, Thermo Scientific™, Marietta, USA) and then centrifuged (HERMLE, refrigerated centrifuge model Z300K, Germany) at 2504 × g for 15 minutes at 4°C to remove spores and other insoluble particles. The recovered supernatant represents the crude enzymatic extract.

Partial Enzyme Purification

In order to prepare a partially purified extract, the enzymatic mixture produced by the fungal strain under the optimal conditions was subjected to precipitation by adding solid ammonium sulfate $(NH_4)_2SO_4$ (AnalaR NORMA PUR®, VWR CHEMICALS Prolabo, Leuven, Belgium) at 20%–70% of saturation. For the two steps of MCE fractionation, the desired amount of salt was gradually added with constant slow stirring at 4°C for 30–60 minutes. The final resulting precipitate was collected after 16-18 hours by centrifugation (2504 × *g*, 1 hour at 4°C) and then re-suspended in a minimal volume of citrate/sodium phosphate buffer (0.1 M; pH 5.2). The saturated extract was introduced into dialysis tubing membrane (Visking size 9-36/32, diameter 28.6 mm, MWCO 12–14 kDa, Medicell Membranes Ltd, London, UK) and dialyzed at 4°C against several changes of the same buffer over 24 hours to remove residual salt. The dilute preparation was concentrated for 2 hours against 50% (w/v) sucrose solution and kept at 4°C. Three parameters were calculated by the following equations to evaluate the efficiency of this step:

- Specific Activity = Total enzyme activity in fraction/Total amount of protein in fraction
- Fold purification = Specific Activity of fraction X/ Original Specific Activity
- Yield (%) = (Total enzyme activity in fraction X/Total original activity)*100

Milk-Clotting Activity

The substrate of the reaction was prepared using skimmed milk at 10% (w/v) in 0.01 M $CaCl_2 \cdot 2H_2O$ solution (pH 6.4). One mL of the enzymatic extract was added to 10 mL of the substrate pre-incubated at 35°C for 15 minutes.[17] The clotting time corresponds to the time elapsing between addition of enzyme and appearance of the first particles of curd on the internal wall of the inclined tube. The curdling activity expressed in the Soxhlet Unit was calculated according to the following equation: MCA (SU/mL) = 2400/T × DF

In the above equation, T is the clotting time (s) and DF is the dilution factor.

Protease Assay

Proteolytic Activity (PA) of the enzymatic extracts was measured according to the method of Anson[18] with slight modifications. The reaction mixture has the following composition: citrate/sodium phosphate buffer (0.1 M; pH 5.2) (0.5 mL); 2.5% (w/v) casein prepared in the same buffer (2.5 mL); enzymatic extract (0.5 mL). The reaction was conducted at 40°C for 30 minutes. Precipitation of the non-hydrolyzed proteins was provided by an addition of 5 mL of pre-chilled trichloroacetic acid at 4% (w/v). The amount of the resulting nitrogen compounds recovered by filtration was determined at 750 nm (UV-1800 Shimadzu Corporation spectrophotometer, Kyoto, Japan) after an addition of diluted Folin-Ciocalteu reagent which specifically reacts with the amino acids tyrosine and tryptophan. One unit (U) of enzyme activity represents the amount of enzyme required to liberate 1 µg of tyrosine from substrate per minute under assay conditions.

Determination of Protein Content

Quantitative estimation of protein was carried out following the method of Bradford[19] based on protein interaction with Coomassie Brilliant Blue G-250 reagent. Bovine serum albumin (Sigma-Aldrich, Germany) was used as a standard. Absorbance of the reaction mixture was measured at 595 nm.

Soft Cheese Making

The crude and the partially purified extracts produced by the strain STIP1 were applied in a trial of soft cheese making. The produced cheese was the Camembert ready for consumption, where the ripening procedure to develop flavour and body characteristics is normally from 10 days at 10–16°C depending on the extent of maturity required.[20]

Preliminary trials were first conducted to precise the volume of each coagulant to be added to a known volume of milk in order to initiate coagulation within 10 minutes.

After pasteurization at 80°C for 2 minutes and cooling to 36 ± 2°C, a precise volume of raw cow's milk (pH 6.4, fat 32%, density 1.027, total solids 11%, with absence of antibiotics) was divided into three equal portions and then inoculated with lactic starter cultures (*Lactococcus lactis* subsp. *lactis biovar. diacetylactis, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis, Leuconostoc* sp., *Streptococcus thermophilus*) and secondary starter cultures (*Debaryomyces hansenii, Penicillium camemberti*, *Geotrichum candidum*, *Penicillium candidium, Staphylococcus xylosus*) (Danisco, France and Chr. Hansen, Denmark).

For the first portion of milk, coagulation was obtained by using the vegetarian rennet MARZYME® (EC. 3.4.23.23) produced by *Mucor miehei* (710–739 IMCU/mL, Danisco, Denmark) at 0.01% (v/v), while for the other volumes of milk, 0.12% (v/v) of the crude extract and 0.066% (v/v) of

the pre-purified extract, were added separately. Prior to their application in cheese making, the fungal extracts were filtered through a sterile Fioroni Syringe filter in PTFE 0.45 µm to remove any traces of spores or mycelium fragments.[21] After completely coagulation according to the desired strength of the curd, 30% to 40% of the whey was evacuated during the cutting step. The obtained curd cubes were poured into rounded pierced plastic containers to mat together as the whey was drained. The moulds were inverted three times to allow further elimination of the whey. The samples were recovered after 24 hours and were then introduced into a brine solution at 20% (w/v) for 5 to 10 minutes. In ripening rooms, the dried cheeses were placed at 13°C with 80-90% relative humidity for 13 days and turned every 4–5 days.

All the physicochemical analyses of the cheeses were performed according to standards methods.[22] Microbiological analyses were carried out under specific culture conditions to detect and enumerate the following bacteria in the pasteurized milk and in the produced cheeses: Total coliforms (Desoxycholate Agar, 37°C, 24 hours), Fecal coliforms especially *Escherichia* spp., *Enterobacter* spp. and *Klebsiella* spp. (Desoxycholate Agar, 44°C, 24 hours), *Clostridium* sulphito-reducing (Meat Liver Agar SR, 46°C, 72 hours) and *Staphylococcus aureus* (Chapman Agar, 37°C, 24 hours).

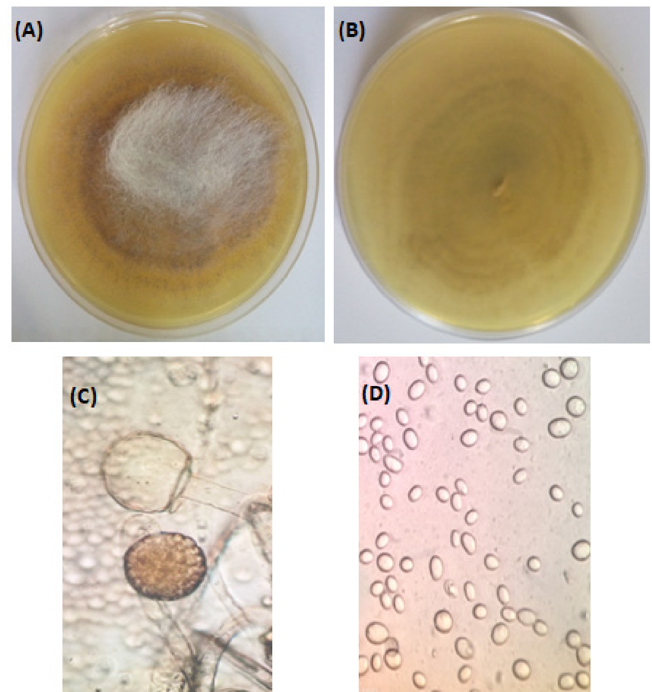

Figure 1. Macroscopic Appearance on Sabouraud Agar (Culture of 5 Days at 28°C; (A): Top, (B): bottom) and optical microscopy images (C, D) used for the identification of the novel isolated strain, × 400.

Results and Discussion

Screening of Fungal Isolates for Milk-clotting Activity

About 20 fungal strains were isolated from the soil samples tested. Depending on the morphological characteristics (size, shape and colony colour etc) of the purified fungal strains, they were principally classified into 5 genera: *Rhizopus, Mucor, Lichtheimia, Penicillium* and *Aspergillus* (Section *Nigri*, *Flavi* etc). According to the results of screening, the most tested fungal strains were able to produce enzymatic extracts with clotting time between 4 and 17 min. Only two fungal strains *Mucor* sp. TIP1 and *Rhizopus* sp. TIP2 were the hyper producers with milk-curdling time of 125 and 145 seconds, respectively. Moreover, the crude extracts produced by both strains were characterized by the best MCA/PA ratios of 3.27 and 2.57, respectively. Thus, *Mucor* sp. TIP1 was selected for furthers studies.

Morphological and Molecular Identification

According to the macroscopic examination of the selected strain developed on Sabouraud Agar at 28°C for 120 hours, the colony of *Mucor* sp. TIP1 is cottony and floccose, pale greyish-brown with the development of sporangia (Figure 1A) while the bottom is colourless (Figure 1B). From the microscopic observation, it has been deduced that long and aseptate hyphae were produced that give rise to sporangiophores which bear spherical to ellipsoidal columella with a sporangium at the apex. No rhizoids, stolons or apophysis were observed. Sporangiophores are hyaline and mostly sympodially branched with long branches erect and shorter branches becoming circinate (recurved). A visible collarette (remains of the sporangia wall) was generally left at the base of the sporangium following its rupture to release spores. Sporangia are spherical (Figure 1C) and spores are smooth, round to ellipsoidal (Figure 1D).

The results of the present study were confirmed by the molecular identification based on the sequencing of the ITS region of rDNA. The selected strain was identified as *Mucor circinelloides* von Tieghem with a specific number from the BCCM-MUL collection: 2095-2047. The PCR product obtained after purification from the electrophoresis gel and sequencing is shown in the Figure 2A. The study of the phylogenetic tree (Figure 2B) revealed that the isolated *M. circinelloides* 2095-2047 had 99% similarity with *M. circinelloides* F5-3-11. The strain was further integrated in GenBank database and it has taken an accession number of MG603064.

Production of the Milk-Clotting Enzyme

Selection of Substrate

The highest enzyme production with a MCA of 271.73 ± 3.6 SU/mL was obtained with wheat bran as the substrate, followed by corncobs and barely bran (Figure 3A), probably induced by its higher protein content (14-16%) compared to other agro-industrial by-products. Results are in agreement with those reported previously.[23-27]

Smaller particle sizes of the substrate in SSF provides large surface area for microbial proliferation and full contact with nutrients, but according to the obtained results, the use of flour for the three substrates induced significant decreases of enzyme production (lost of 30–50% of the activity) as the consequence of limited mycelial growth, which can be explained by the reduction of heat transfer, substrate

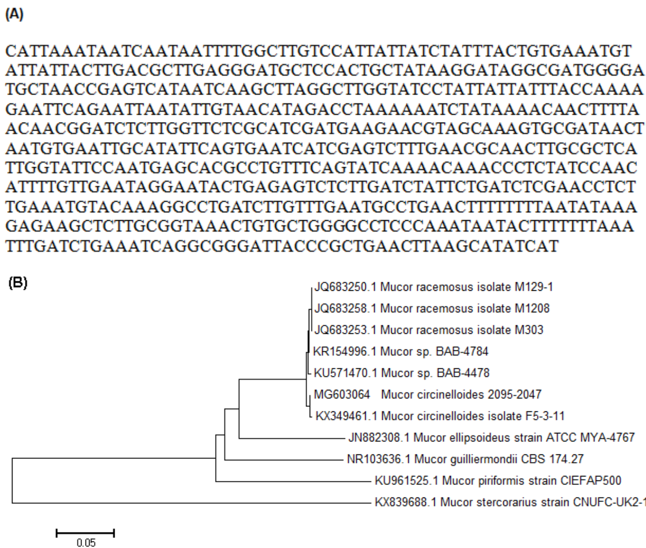

Figure 2. Sequence of the ITS region of *Mucor* sp. TIP1 (*M. circinelloides* 2095-2047) (A) and partial ITS rDNA sequence-based phylogenetic neighbour-joining tree showing the phylogenetic relationship of the isolated strain relative to other strains of the *Mucor* genus (B).

consumption and gas exchange (O_2 and CO_2) during fermentation.[9,10,28,29]

Choice of the Mineral Medium

Microbial proteases production can be enhanced by the supplementation of mineral salts, which also play a vital role for microbial growth because of their involvement in metalloenzymes or as enzyme activators.[30] The results illustrated in Figure 3B indicate that more than 29% increase in enzyme activity was obtained when distilled water was replaced by M-9 solution, while Czapek-Dox medium gave the lowest activity with 36% decrease. Similar findings were noted for alkaline protease production by *Rhizopus oryzae*[15] and *Beauveria felina*[31] on the same substrate. The ratio MCA/PA increased from 5.71 for the first step to 8.43.

It is very important to note that the use of distilled water improved the MCE production compared to M-15, SM and Czapek Dox solutions, which can be induced by the selected pH. According to reports of Ali,[32] the pH of the culture medium greatly affects the availability of certain metallic ions, permeability of membranes and enzymatic activity. Given that the M-9 medium induced an increase of the MCA with a fold of 1.222 compared to distilled water, it was selected for the next experiences.

Effect of the Substrate Amount

In SSF process, the selection of substrate and optimization of its concentration play an important role in yielding the higher growth rates of microbes and products formation.[9] As shown in Figure 3C, a quantity of 10 g of wheat bran was found optimal for the enzyme production (MCA = 549.7 ± 7.32 SU/mL, MCA/PA = 10.50). Significant reduction in enzyme activity was detected with increasing the substrate amount in the flasks, which probably was induced by the decrease of heat and oxygen transfer. The amount of wheat bran introduced

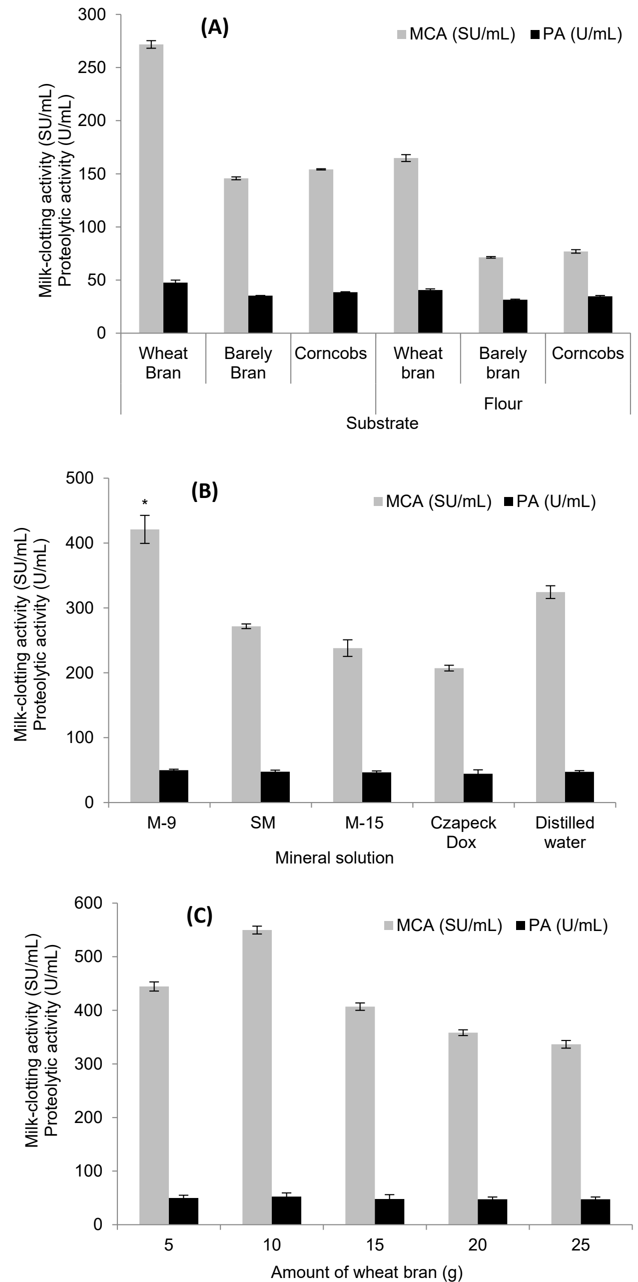

Figure 3. Effect of agro-industrial by-products (A), saline media composition (B) and wheat bran amount (C) on MCE production by *M. circinelloides* (The results were shown as mean value ± SD, * $P < 0.05$).

into the 250 mL Erlenmeyer flasks takes up a large space (bed depth increases with the amount of substrate), which limits the growth of mycelium and consequently the enzyme production.

Effect of Additional Carbon Sources

Among the various external carbon sources tested, galactose, glucose and pods of carob powder added at 1% (w/w) to wheat bran (10 g) were found significantly as the best sources of carbon for the enzyme production, where the MCA enhanced to reach 571.43 ± 7.67 SU/mL for galactose (Figure 4A), while the other sources did not have an effect (beans of carob powder) or they induced the decrease of production

compared to the control. The milk-curdling activity of proteases produced under SSF process by *Rhizomucor nainitalensis*[27] and *A. oryzae*[29] was increased with an addition of 0.4% and 1% of glucose, respectively, to the medium. It can be noted that the higher ratio of MCA/PA (14.36) was obtained when using galactose, induced by the decrease of the proteolytic activity, where only glucose and fructose enhanced this activity compared to the control.

Effect of Additional Nitrogen Sources

An important factor in the growth and production of enzymes by microorganisms is the availability of nitrogen, which can be obtained from organic or inorganic sources.[27] According to the results represented in Figure 4B, the enzyme yield and activity of the MCE produced by *M. circinelloides* were significantly lower than the control for all organic sources. Ammonium nitrate (NH_4NO_3) increased the MCA, but ammonium sulfate provided the maximum production with a significant effect to reach 562.77 ± 15.0 SU/mL. The same results were reported by Negi and Banerjee,[24] while other findings revealed that addition of inorganic nitrogen sources gave poor production of MCEs, which affect the ratio C/N, more than organic nitrogen sources.[29,33,34] The higher yield of extracellular MCE produced by *A. oryzae,*[29] *Rhizomucor nainitalensis*[27] and *Rhizomucor miehei*[35] was recorded in wheat bran supplemented with 0.8% of beef extract, 1.5% and 2% of casein, respectively.

Effect of Incubation Time

The incubation period required for maximal production of the enzyme depends on two factors: the microorganism used and the cultural conditions. As shown in Figure 5, a steady increase in enzyme activities can be noted up to 96 hours where the peak MCA was observed, but with prolonged incubation, the enzyme activities became lowest probably due to the reduction of nutrients, enzyme denaturation and deactivation by others metabolites.[15] It is also possible that even the development of mycelium causes the reduction of inter-particle space which reduces heat transfer, gas exchange and water content.[9,28]

Rhizomucor miehei,[36] *M. thermohyalospora* and *R. azygosporus*[25] were also cultured for 96 hours to obtain maximum amounts of milk-clotting proteases on solid cultures. Whereas, rennin-like enzymes produced by *Rhizomucor miehei*[26] and *M. circinelloides*[23] exhibited the highest activity after 72 and 120 hours of incubation, respectively.

Partial Purification

The crude extract produced by *M. circinelloides* was fractionated using 20%-70% ammonium sulfate saturation with 84% recovery and 2.48 fold purification (Table 1), where 66.14% of contaminating proteins were eliminated.

The cumulative yield of enzyme activity in the partially purified extract exceeded the initial yield to reach 105%. Generally, during the purification process, yield is expected to be lowered induced by the fractionation methods used

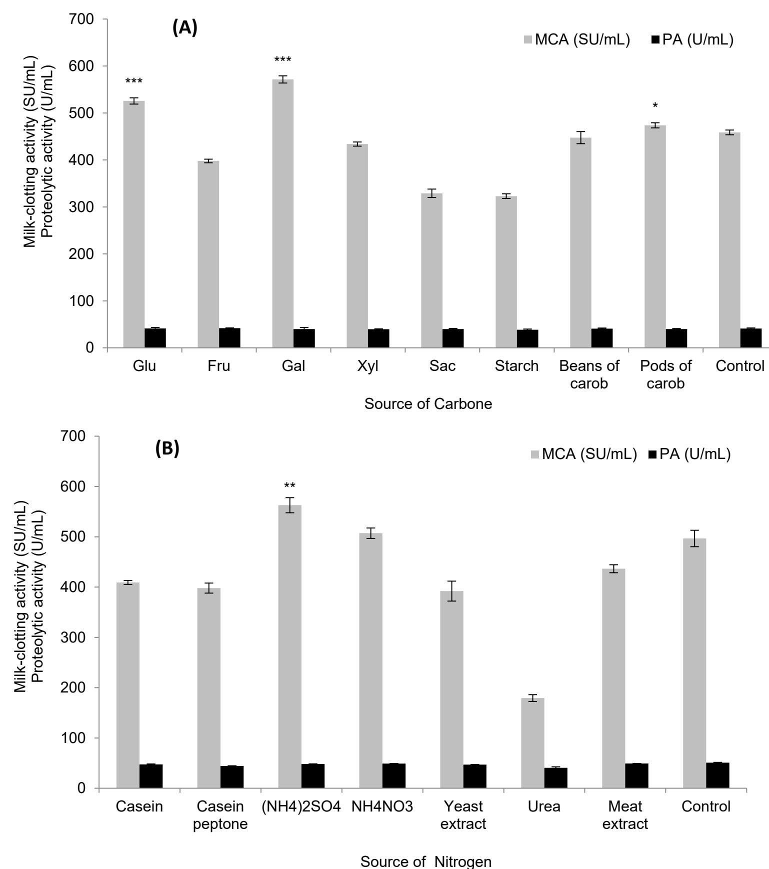

Figure 4. Influence of additional carbon (A) and nitrogen (B) sources on MCE production by *M. circinelloides* (The results were shown as mean value ± SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

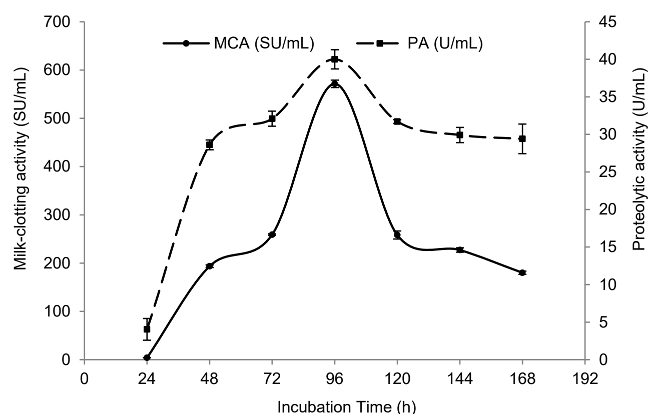

Figure 5. Effect of Incubation Time on the Production of MCE by *Mucor circinelloides*. The results were shown as mean value ± SD.

to obtain a pure enzyme. This result is probably due to the elimination of ammonium sulfate and the presence of one or more inhibitors in the crude extract which were removed during dialysis. The same observations were noted by Kumar et al.[37] After this step, the degree of purification enhanced to achieve 6.233 and the percentage of eliminated proteins reached 83.154% from total protein contents of the crude extract.

It's well known that a high MCA/PA ratio is required for considering an enzyme as rennin substitute to minimize several disadvantages during cheese making (lower cheese yield, incorporation of lipids in the coagulum, bitter taste and abnormal fragrances).[2,3,7] The ratio MCA/PA of the pre-purified enzyme of *M. circinelloides* enhanced from 14.36 to 303, which resulted from elimination of non-specific proteases and improvement of the MCA to achieve 16000 SU/mL. The effect of this improvement was visible during coagulation of the skimmed milk, where the volume of whey was reduced and the amount of curd improved as a function of the partial purification steps.

Camembert Cheese Making

From the obtained results (Table 2), slight differences were noted between the properties of cheese made by the vegetarian rennet MARZYME® and that produced by the fungal extracts of *M. circinelloides*. The physicochemical analyses were compatibles with the internal standards of the production unit and the codex CXS 276-1973[20] for Camembert, except for the fat (30.5%) and fat/dry matter (56.1%) of cheese produced by the crude extract which were superiors to the typical values.

The cheese yield obtained with the crude extract of *M. circinelloides* was lower compared to that provided by the microbial rennin of *M. miehei* (a difference of 22%). This variation was induced by the presence of non-specific proteases and possible lipases (lost of proteins and fat in the whey) in the crude extract. The use of partially purified extract characterized by a high MCA and a low PA, improved the cheese yield which was very close to that obtained by the vegetarian rennet (a difference of 2.5%).

The microbiological analyses showed the total absence of contamination in the pasteurized cow milk used in cheese production which revealed the success of the pasteurization process. Likewise, the absence of *Staphylococcus aureus* and *Clostridium* sulfito-reducing has been recorded in all produced cheeses. Concentration of total coliforms and fecal coliforms in cheeses were less than the critical values of 100 CFU/mL and 10 CFU/mL, respectively.[38] In view of the fact that hygienic conditions have been respected during cheese making, the fecal contamination can be explained by the ability of coliforms to build up rapidly in moist milk residues and in biofilms on milking equipment and thus contaminate the end product.[38]

The sensory analysis of the three Camembert cheeses by about 15 panelists from the Department of Food Technology (University of Boumerdes) and Department of Biological Sciences (University of Bouira) has revealed the principal characteristics summarized in Table 3.

No gas holes were formed in the three produced cheeses (Figure 6), which represents one of the specific characteristic of the Camembert cheese.[20] It is well known that during ripening, biochemical and enzymatic reactions (glycolysis, lipolysis, proteolysis) occur which result in the development

Table 1. Partial Purification Steps of the MCE Produced by *Mucor circinelloides*

Purification Steps	Total MCA (SU)	Total Protein (mg)	Specific Activity (SU/mg)	Fold Purity	Yield (%)
Crude extract	114285	58.50	1953.60	1.00	100
$(NH_4)_2SO_4$ fractionation (20–70%)	96000	19.81	4846.53	2.48	84
Dialysis at pH 5.2	120000	9.85	12176.56	6.23	105

MCA: milk-clotting activity.

Table 2. Physiochemical Properties of the Three Camembert Cheeses

	pH	Dry Matter (%)	Fat (%)	Moisture (%)	FDM (%)	Yield (g/L)
Chemical Standards	≤ 5.5[a]	Minimum 38–48[20]	20–28[a]	40–60[a]	45–55[20]	/
Cheese produced by:						
Vegetarian rennet MARZYME®	5.23	52.31	25.00	47.69	52.42	120
Crude extract of *M. circinelloides*	4.97	54.36	30.50	45.64	56.10	93.6
Pre-purified extract of *M. circinelloides*	4.90	51.16	28.00	48.84	54.73	117

FDM: fat in dry matter.
[a] Standards of the production unit.

Table 3. Sensory Analysis of the Produced Camembert Cheeses

Parameter	Cheese Made With		
	Vegetarian Rennet MARZYME®	Crude Extract of *M. circinelloides*	Pre-purified Extract of *M. circinelloides*
External appearance (Crust)	Bloomy white, regular, thin	Bloomy white, regular, thin	Bloomy white, regular, thin
Internal appearance	Light yellow colour, Smooth, compact, bright	Off-white colour, Smooth, dissociated	Light yellow colour, Smooth, compact, bright
Odour	Camembert	Camembert, pungent (rancid)	Camembert
Taste	Slightly salty, buttery	Salty, weakly sour, musty and bitter	Slightly Salty, buttery, milky
Texture and mouth-feel	Soft, moist, supple	Gluey and hard	Soft, supple, moist

of flavour, physical properties (melt, stretch), texture and changes to the body of the cheese.[1,2] For the soft cheese Camembert, the repining starts from the surface to the center.[20] In the case of cheese prepared by the crude extract of *M. circinelloides*, it has been observed that after 13 days, the repining was incomplete, where the body was always white with a solid texture when pressed by the thumb. These results are probably due to the blocking of enzymes synthesized by the ripening ferments, induced by the presence of inhibitors in the crude extract and/or theirs hydrolyze by some proteases produced by the strain. Also, the others defects of taste and odour developed in the cheese can be explained by the non-specific action of proteases and lipases of the crude extract on the other caseins of milk (especially casein-α and β) and on milk fat to produce fatty acids respectively.

Application of the partially purified extract in the cheese making allowed correction of the defects previously obtained with the crude extract. The majority of the tasters found that sensory characteristics of the two cheeses obtained by the commercial rennet and the pre-purified extract of *M. circinelloides* were similar, where some of which preferred the last one especially for the taste, a little moister, melting and suppler compared to that obtained by MARZYME® rennet.

Conclusions

Production of rennin-like enzyme by the new soil isolate *M. circinelloides* MG603064 was carried out in solid cultures.

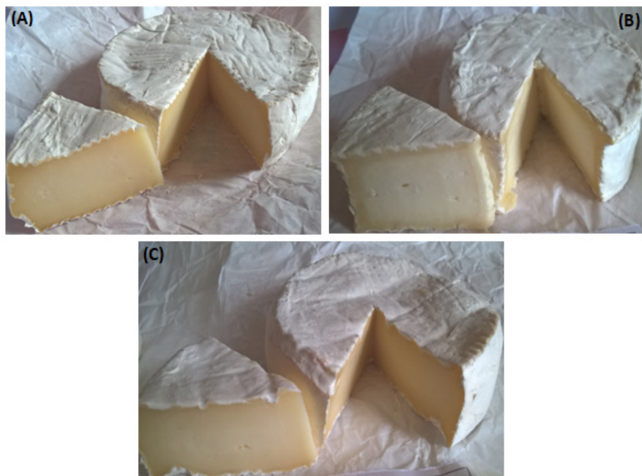

Figure 6. Samples of the soft cheese "Camembert" produced by the commercial rennet MARZYME® (A), the crude enzymatic extract (B) and the partially purified extract of *M. circinelloides* (C).

After the optimization of different parameters of the SSF process, the milk-clotting activity of the crude extract was increased from 192 SU/mL to achieve 571.43 ± 7.67 SU/mL (clotting time of 42 seconds) which represented an increase by about 3-fold. The crude extract exhibited a low proteolytic activity of 39.80 ± 0.40 U/mL and a ratio MCA/PA of 14.36. The milk-curdling activity and the ratio MCA/PA were strongly enhanced to reach 16000 SU/mL and 303, respectively, after a partial purification step using $(NH_4)_2SO_4$ fractionation and dialysis.

The obtained results of the cheese making trials are very satisfactory, which makes it possible to predict that the purified MCE of *M. circinelloides* can be considered as a new substitute for calf rennet in the cheese manufacture. In order to achieve this goal, the present study must be completed by further optimization and purification to increase the MCA of the purified coagulant and its application in the production of different varieties of cheese.

Authors' Contributions

All experiments, the manuscript writing and its finalization were carried out by SB. KB was involved in the molecular identification of the strain; and FN-F supervised the work.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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