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Production of White Soft Cheese Using Fungal Coagulant Produced by Solid State Fermentation Technique

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Abstract: The solid state fermentation (SSF) technique was used for preparation of a fungal rennin from a strain of *Rhizomucor miehei NRRL* 2034. Fungal rennin was isolated, partial purified and use as adequate calf rennet substitute. UF-white soft cheese samples were laboratorial manufactured and stored for two months at refrigerator temperature (7±2°C). The results revealed that one ml fungal rennin/100 ml milk gave the close properties to calf rennet control cheese. Chemical and organoleptic properties of all cheese samples were periodically evaluated when fresh and after 15, 30 and 60 days of cold storage. Experimental cheese (E) had higher values of soluble nitrogen (SN), total volatile fatty acids (TVFAs) and tyrosine and tryptophan than control cheese. Furthermore, experimental cheese seemed to have soft body and smooth texture as well as a desirable taste during cold storage at for 2 months.

Key words: Rennin substitute · *Rhizomucor miehei NRRL*2034 · Solid state fermentation technique · UF-white soft cheese

INTRODUCTION

The recent increasing in global cheese production coupled with the sharp scarcity on calf rennet which obtained from the fourth stomach of suckling calves; make a growing tendency for searching new rennet substitutes. The active milk-coagulating enzyme present in calf rennet extract is an acidic protease which is donated as calf chemotrypsin; aspartyl proteinase, EC 3.4.23.4 [1]. Today, only 20-30% of the world demand for milk-clotting preparation was be covered by calf rennet [2]. Many successful attempts had been done for producing milk clotting enzymes from other sources such as plants [3-5] or microorganisms [6-9]. Various microorganisms are known as producers of rennet-substitute such as *Aspergillus oryzae, Endothia parasitica*, *Mucor circinelloides, Rhizomucor miehei* and *Rhizomucor pusillus* [7, 8, 10]. On the other hand, various cheese types were made from microbial coagulants [11, 12, 13]. Through different microorganisms; *Rhizomucor miehei* is considered as a good source of calf rennin substitute. Many researches were conducted for its production conditions, the clotting ability, various properties of its milk clotting enzyme and its application on cheese manufacture [2, 14-17]. The protease produced by *Mucor miehei* mold (EC 3.4.23.10) is the preferred substitute for true calf rennet due to its specificity in splitting similar peptide bonds in kappa-casein, high ratio of milk coagulating activity, identical calcium requirements and good cheese quality [1]. The protease of *Mucor miehei* is an acid-aspartate protease having a molecular weight of about 38.000. The molecule consists of a single polypeptide chain with high similarity to calf rennet in 3-D-structure. Aspartic proteases produced from *Mucor miehei* have less heat stability which makes it more sensitive for heating during cheese manufacture. So, limited proteolytic action and subsequently no bitter taste were occurred [2].

In Poland, fungal coagulant obtained with *Rhizomucor miehei* are already used in the industrial cheese production and successfully applied in Camembert, Edam and Cheddar cheeses [13]. Nowadays,

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a lot of studies were done for estimating the ideal production conditions to prepare rennet- substitute enzyme from *Mucor miehei*. Solid State fermentation (SSF) technique is one of the recent techniques particularly suitable for milk clotting enzyme production. It has a potential advantages like; low energy consumption, high yields, low environmental impact, differential expression of metabolites and requirement of less expensive technology [6, 10, 17]. UF-white soft cheese is the main Egyptian soft cheese where UF- technique is almost applied in all Egyptian factories; they used exported and expensive microbial rennet. Therefore, the aim of this study was applying of solid state fermentation (SSF) technique in preparation of milk clotting enzyme from *Rhizomucor miehei* NRRL 2034 as adequate calf rennet substitute. And subsequently, using this fungal prepared enzyme in the production of UF-white soft cheese and evaluate some properties of resultant cheese during cold storage.

MATERIALS AND METHODS

Raw Materials: Wheat bran was obtained from local market, Cairo, Egypt. Fresh buffalo's milk and milk retentate were obtained from Animal Production Institute, Ministry of Agriculture, Egypt. Calf rennet powder (HALA) had enzyme productivity equal 150000 Soxhelt units/g, as well as starter cultures (*Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*) were obtained from Chr. Hansen's Lab., A/S Copenhagen, Denmark. *Rhizomucor miehei* NRRL 2034 was obtained from Northern Regional Research Lab., Peoria Illinois, USA. The fungal culture was periodically subculture and maintained on potato dextrose agar medium.

Inoculums Preparation and Solid State Fermentation (SSF): The inoculum for SSF was prepared by scrapping the surface of five days old cultures on potato dextrose agar (PDA) medium with sterile distilled water. The spore suspension was used as inoculum. Wheat bran; as industrial residue; was used as substrate in SSF for milk clotting enzyme production. Wheat bran was dried at 60°C for one hour, grinded and distributed in Petri dishes, moistened with distilled water, autoclaved for 30 min, inoculated with spore suspension (1.8×10^8 cfu/g) so incubated at 40°C for 3 days [18].

Large Scale Production of Milk Clotting Enzyme: The standardized wheat bran medium with 50% moisture content was evenly distributed in aluminum foil trays ($20 \times 25 \times 5$ cm^3). The trays were sterilized in the autoclave for 30 minutes. The medium was inoculated with the actively growing starter cultures of *Rhizomucor miehei* NRRL 2034 and the cultivation was carried. Ten trays of each run were loaded at a time for the enzyme production under the standard conditions.

Enzyme Extraction: After the incubation period, the enzyme in a known quantity of solid product was extracted with distilled water (1:10 w/v) under shaking at 150 rpm for 2h at 30°C. The obtained filtrate was centrifuged at 4000 rpm for 10 min. The supernatant was used as crude enzyme source for milk clotting activity.

Determination of Milk Clotting Activity (MCA) and Protease Activity (PA): The method of Greenberg [19] was used for determining the MCA and PA. The activity of milk clotting enzyme was expressed in Soxhelt units. One milk clotting unit is defined as the amount of enzyme preparation which clots 1ml of skim milk in 40 min. at 35°C. One unit of protease activity was defined as the amount of enzyme, which released 1µg of amino acid equivalent to tyrosine under the assay conditions.

Detection of Aflatoxins in Fungal Enzyme: Aflatoxins B1, B2, G1, G2, Ochratoxina and Zearalenone were detected in enzyme preparation sample according to AOAC [20].

Milk Clotting Enzyme Application:

Preliminary Trail: Salted fresh buffalo's milk (3.0 % NaCl) was heated at 75°C and cooled to 38°C, then divided into five equal portions. One part was calf-renneted at rate of 1mg/100ml milk serve as a control (C). The fungal coagulant was added at the rate of 0.5, 1.0, 1.5 and 2.0 ml/100 ml milk to create four treatments. All treatments were incubated at 38°C until complete coagulation. Coagulation time, curd syneresis, yield and body properties as well as total acceptability were estimated to determine the appropriate concentration of the fungal enzyme which will be used to produce the UF-white soft cheese in this study.

UF-white Soft Cheese Manufacture: UF-soft cheese was made according to the method described by Renner and Abd El-Salam [21]. Pasteurized buffalo milk retentate was divided into two equal portions. The first part was renneted using calf rennin-coagulate (1g/100 kg) as a control (C). The second part was renneted using the applicable ratio of fungal coagulant (1.0 %) as experimental treatment (E). Milk retentate dispensed into

plastic bags (500 ml) and held at 38°C until a uniform coagulum was formed. All cheese samples were stored at 7±2°C and analyzed when fresh and then after 15, 30 and 60 days of storage. Three replicates were done.

Analytical Procedures of Cheese Samples: Total solids (TS), fat, total nitrogen (TN) and soluble nitrogen (SN) contents of cheese samples were determined according to AOAC [20]. The pH value was measured using digital pH meter (HANNA, Instrument, Portugal) with glass electrode. The spectrophotometric method of Vakaleris and Price [22] was used for measuring tyrosine and tryptophan contents in cheese samples. Total volatile fatty acids (TVFAs) were determined as given by Kosikowski [23].

Sensory Evaluation: The sensory properties of the resultant cheese samples were evaluated periodically by 20 regular-score panelists when fresh and after 15, 30 and 60 days of cold storage for flavor (50 points), Body and Texture (40 points) and Appearance (10 points). All acceptability recorded 100 points.

Statistical Analysis: The obtained data were statistically analysis using SAS [24], the statistic version 8th Ed. Differences among means were identified using Duncan multiple range test.

RESULTS AND DISCUSSION

Ideal Conditions of the Large Scale Production of Milk Clotting Enzyme: The obtained results showed that wheat bran yielded the highest MCA of *Rhizomucor miehei* NRRL 2034; and the moisture content of enzyme preparation was (50%), the ideal incubation temperature was 40°C and incubation period was 3 days and its optimum pH was 5. The milk clotting activity/protease activity (MCA/PA) equal 11.9 as recorded in previous study [17].

Detection of Aflatoxins: Aflatoxins B1, B2, G1, G2, Ochratoxina and Zaralenone were detected in the fungal milk clotting enzyme obtained from *Rhizomucor miehei* NRRL 2034. The data indicated that the sample is toxins free. So it can be used with high safety as rennin substitute in coagulation of milk and manufacture of cheese. Milk coagulating enzyme obtained from *Rhizomucor miehei* has a sufficient toxicological data set to support its safety [25]. Moreover, this enzyme has safe use in the Canadian food supply in the manufacture of cheese. The Joint FAO/WHO Expert Committee for Food Additives (JECFA) in 1974 established an acceptable daily intake of 'not specified' for fungal rennet from *Mucor miehei*. These substances have a very low toxicity and do not pose a health hazard. Milk coagulating enzyme from *R. miehei* is already permitted for use in Canada as a food enzyme in various types of cheese, sour cream, dairy-based flavoring preparations and hydrolyzed animal, milk and vegetable protein [25].

Determination of the Appropriate Fungal Enzyme Concentration for Experimental Cheese: Data presented in Table 1 showed the properties of the preliminary white soft cheese samples. Different concentrations of fungal milk clotting enzyme (0.5, 1.0, 1.5 and 2.0 ml/100ml milk) isolated from *Rhizomucor miehei NRRL 2034* was applied to determine the adequate percent will be used in this study. As shown in Table 1, it could be concluded that using 1ml milk clotting preparation /100 ml milk was more suitable for manufacture of white soft cheese and the properties of the resulted cheese samples was very closed to that produced by calf rennin. Total acceptability of the experimental samples was 95% against 92% for calf rennet-sample. So using 1ml fungal enzyme /100 ml milk was selected in all subsequent experiments.

Properties of the Adoptive Uf-white Soft Cheese: Table 2 reveals the gross chemical composition and some ripening parameters of fresh and stored UF-white soft cheese made with fungal rennet-like-enzyme obtained from *Rhizomucor miehei NRRL 2034* (E) ; in comparison to control cheese made with calf rennet (C) through 60 days of storage at cold storage (7± 2°C). For gross composition contents, no pronounced differences were observed in TS, TN and fat percent in both fresh and stored cheese samples; where C-samples gained 33.67, 2.65 and 14.5% respectively against 31.77, 2.53 and 14.0 % for E-samples. These differences had no viable effect upon cheese in the production scale. The changes of all data through storage were logically remarkable and accepted as recorded by Abbas *et al.* [11] and Ismail *et al.* [26].

The pH values presented in Table 2 reflected the action of both cheese culture and the additives (calf rennet and fungal enzyme). It could be noticed a gradual decreased in pH values during ripening in both cheese samples; but the rate of decrease was more pronounced in E-cheese. C-cheese had 5.58, 5.51, 5.36 and 5.10 when fresh and after 15, 30 and 60 days of ripening versus 5.47, 5.78, 5.55 and 5.01 respectively for E-cheese.

Table 1: Properties of white soft cheese samples made with different concentrations of fungal milk clotting enzyme isolated from *Rhizomucor miehei* NRRL 2034.

Coagulants %	Coagulation time (min)	Cheese yield over 4 hr (g/100ml)	Curd syneresis (ml)	Body and Texture properties	Total acceptability (100)
0.5 *	55	34.23	52	very soft	90
1*	50	29.27	56	Soft	95
1.5*	45	32	57	Soft	90
2*	40	30.41	59	soft	91
C **	50	28.11	62	firm	92

* 1ml / 100 ml milk v/v)

* *1mg/100 ml milk (w/v)

Table 2: Cross chemical composition and some ripening parameters of fresh and stored UF-white soft cheese made by fungal rennin-like enzyme from *Rhizomucor miehei* NRRL 2034 in comparison with control cheese made by calf rennet through 60 days of cold storage.

Treatment	Storage period(days)	pH	T.S %	T.N %	Fat %	TVFA *	S N %	SN/TN	Tyrosine **	Tryptophan **
Control cheese (C)	0	5.58^{A}	33.67^{D}	2.65^{CD}	14.5^{F}	14.5^{E}	0.22^{F}	0.083^{E}	39.5^{E}	37.08^{D}
	15	5.51^{A}	33.86^{D}	2.68^{CD}	14.6^{E}	16.2^{D}	0.24^{E}	0.0895^{DE}	45.94^{D}	38.02^{D}
	30	5.36^{AB}	34.9^{B}	2.84^{AB}	14.8^{D}	20.9^{C}	0.27^{D}	0.095^{D}	51.1^{C}	40.90^{BC}
	60	5.10^{BC}	35.1^{B}	2.91^{A}	14.9^{C}	30^{B}	0.31^{C}	0.106^{C}	56.19^{B}	41.92^{AB}
Experimental cheese (E)	0	5.47^{B}	31.77^{E}	2.53^{D}	14.0^{G}	21^{C}	0.28^{D}	0.111^{C}	40.93^{E}	38.15^{D}
	15	5.78^{C}	31.68^{E}	2.7^{BC}	14.0^{G}	22.5^{C}	0.31^{C}	$0.115B^{C}$	46.6^{D}	39.89^{C}
	30	5.55^{D}	34.44^{C}	2.85^{AB}	15.0^{B}	29.5^{B}	0.35^{B}	0.123^{B}	55.375^{B}	40.45^{C}
	60	5.01^{E}	36.90^{A}	2.91^{A}	16.0^{A}	40.0^{A}	0.42^{A}	0.144^{A}	60.08^{A}	42.07^{A}

* Expressed as ml 0.1 N NaOH/10 g cheese sample. **Expressed as mg/100 g cheese sample.

-Each treatment was an average of three replicates -Means with the same capital letters are not significantly different (P=0.05).

Table 3: Organoleptic properties of fresh and stored UF- white soft cheese made with fungal rennin-like enzyme from *Rhizomucor miehei* NRRL 2034 in comparison with control cheese made by calf rennet through 60 days of cold storage.

Treatment	Storage period(days)	Flavor (50 p)	Body and Texture (40p)	Appearance (10p)	Total acceptability (100)
Control cheese ©	0	42.80^{CD}	35.7^{AB}	9.2^{AB}	87.7
	15	41.80^{D}	33.80^{B}	8.3^{AB}	83.9
	30	45.10^{ABCD}	35.70^{AB}	8.2^{B}	89
	60	46.60^{AB}	37.50^{A}	9.0^{AB}	93.1
Experimental cheese (E)	0	45.40^{ABCD}	36.70^{AB}	9.2^{AB}	91.3
	15	43.30^{BCD}	34.40^{B}	8.5^{AB}	86.2
	30	45.90^{ABC}	36.60^{AB}	8.6^{AB}	91.1
	60	47.0^{A}	37.70^{A}	9.3^{A}	94

-Means with the same capital letters are not significantly different (P=0.05).

Total volatile fatty acids contents (TVFAs) showed great differences in both cheese samples (C and E). Control-cheese gained lower content of TVFAs either fresh or during ripening (Table 2). They possessed 14.5, 16.2, 20.9 and 30.0 ml 0.1N NaOH/ 10g cheese against 21, 22.5, 29.5 and 40.0 for E-cheese samples at fresh and after 15, 30 and 60 days of ripening respectively. These variations could be related by the higher lypolitic activity of fungal enzyme. These results are in accordance as mentioned by Abbas *et al.* [11] and Abd-Rabou and El-Senaity [16]. Reps *et al.* [13] mentioned that greater amount of free fatty acids were recorded for camembert cheese made by *Rhizomucor miehei NRRL 2034* coagulant enzyme. Moreover, a great content of free unsaturated-fatty acids was also observed in E-cheese. Data in Table 2 also illustrated that the contents of SN in both cheese samples (C, E) during ripening for 60 days. It could be noticed gradually increased in SN contents in both cheese samples as a result of proteolysis. There were obvious differences in the proteolytic action of the two coagulants. Fungal preparation had more proteolytic activity and hydrolyzed more peptides bands and liberated more free amino acids and more soluble nitrogen [11, 27]. Control cheese samples had 0.22, 0.24, 0.27 and 0.31 % SN contents versus 0.28, 0.31, 0.35 and 0.42 % for E-cheese respectively. Abd-Rabou and El-Senaity [16] reported that Edam cheese produced by *Rhizomucor miehei milk clotting enzyme* had more SN and NPN more than control. On the other hand, Reps *et al.* [13] indicated that the amount of milk N-compounds and fat in whey (obtained by mucor proteinase preparation) was higher than that obtained in coagulation

with rennin during the manufacture of camembert cheese. After 2 weeks-ripening; the content of N-compounds in cheese produced by *Rhizomucor* proteinase was higher than in rennin-cheese. Their obtained results explicitly indicate that the protein degradation process is more intense in cheese produced with fugal enzyme and thus ripens faster [13]. For tyrosine and tryptophan contents, the same table revealed the changes in their values freshly and during storage. E-cheese had higher values through all intervals in comparison with C-cheese as the same trend for soluble nitrogen. Fresh C-cheese had 39.5, 37.08 mg/100g against 40.93, 38.15 tyrosine and tryptophan for E-cheese. During ripening the differences were more pronounced where C-cheese gained 56.19, 41.92 mg/100g after 60 days versus 60.08, 42.06 for tyrosine and tryptophan for E-cheese. These results are in accordance with those reported by Abbas *et al.* [11] and Abd–Rabou and El-Senaity [16].

Table 3 reflects the sensory properties of UF-white soft cheese samples prepared by calf and fungal coagulants. Data revealed the acceptability of E-cheese samples compared with C-cheese samples. Fresh C-cheese seemed to be firm with acceptable body and texture and clean flavor. While, E-cheese samples had a soft spreadable body and pronounced flavor with more acceptability. No flavor defects or no bitter tastes were observed in both E and C-cheeses during all intervals of ripening. Generally, ripening period improved all the organoleptic properties of both C-and E-cheese samples for the proteolytic and lypolitic actions. For flavor assessment; C-cheese samples possessed 42.8, 41.8, 45.10 and 46.6 points when fresh and after 15, 30 and 60 days against 45.4, 43.3, 45.9 and 47 points for E-cheese samples respectively. On the other side, body and texture data showed that C-cheese samples gained 35.7, 33.8, 35.7 and 37.5 points Vs 36.7, 34.4, 36.6 and 37.7 for E-cheese samples respectively. Total acceptability of C-cheese samples after 60 days was 93.1 points and 94 points for E-cheese. Abbas *et al.* [11] confirmed these data. In the production of Camembert, Edam and Cheddar cheeses, the curd obtained with Rhizomucor proteinase was slightly less firm than that obtained with rennin preparation [13].

CONCLUSION

In this study, it can be successfully applied the enzyme preparation obtained from *Rhizomucor miehei* NRRL 2034; produced by solid state fermentation technique; in the production of UF-white soft cheese at commercial production scale in Egyptian factories; in spite of the exported expensive enzyme preparations.

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