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Over Production of Milk Clotting Enzyme from *Rhizomucor miehei* Through Adjustment of Growth Under Solid State Fermentation Conditions

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Abstract: The present study introduces a novel biotechnology for cost effective and economically feasible production of natural fungal rennin in Egypt to fulfill the local requirements of cheese industry instead of the calf rennet that is highly expensive and imported from abroad. In this study, *Rhizomucor miehei* NRRL 2034 exhibited the highest enzyme productivity under solid state fermentation. Among twelve industrial by-products used as nutrient substrates for *Rhizomucor miehei* growth under solid state fermentation, wheat bran yielded the highest milk clotting activity (33350 SU/g fermented culture). The optimum conditions for milk clotting enzyme production were moisture content of 50%, incubation temperature 40°C, incubation period 3 days and inoculums size of 1.8×10^8 CFU/g wheat bran. Addition of whey powder as a nitrogen source at 2% increased the enzyme productivity about 42% compared to the control medium. Wheat bran particle size more than 300 µm was the most suitable size for the highest production of the enzyme. The maximum milk clotting activity was achieved by using mineral solution as a moistening agent for wheat bran. Large scale production of the enzyme in tray showed the maximum activity when 125 grams wheat bran were distributed in an aluminum foil trays (20×25 ×5 cm^3) under the optimum cultural conditions. Milk clotting activity achieved in trays was 63550 SU/g fermented culture with high milk clotting activity to proteolytic activity ratio 10:1. Biochemical characterization of the enzyme showed the highest milk clotting activity at 60°C reaction temperature, pH 5, 0.05M $CaCl_2$ and 9% skim milk.

Key words: Milk clotting enzyme, *Rhizomucor miehei*, Solid state fermentation.

INTRODUCTION

Rennet is an aspartate protease enzyme used in cheese making (Kumar *et al*., 2005). The coagulation of milk casein by rennin takes place in two phases, the enzymatic and non-enzymatic phases. In the enzymatic phase, casein is hydrolyzed by rennin to form Para-casein which subsequently becomes a curd in non enzymatic phase under appropriate temperature and concentration of calcium ions (Fox *et al*., 2000). Calf rennet is obtained from the stomach of unweaned calves (Dutt *et al*., 2009). Previously, calf rennet was extensively used in cheese industries. An increase in demand for global cheese production coupled with shortage in calf rennet production as well as the associated ethical issues, have necessitated a search for alternative rennet substitutes, among which are microbial milk clotting enzymes (Tubesha and Al-Delaimy 2003).

Microorganisms generally recognized as efficient milk clotting enzyme producers are such as *Rhizomucor miehei*, *Rhizomucor pusillus* (Seker *et al*., 1999; Nouani, *et al*., 2011), *Aspergillus oryzae*, *Endothia parasitica* (da Silveira *et al*., 2005) and *Rhizomucor baciliformis* (Areces *et al*. 1992). A number of researches have been accomplished on an investigation of calf rennet substituted enzymes from microorganisms (Hashem, 2000). However, fermentation conditions as well as medium components reported for the rennet production by those organisms are widely varied. Microbial milk clotting enzymes, which constitute about 33% of total protease utilization, largely replace calf rennet for milk clotting in cheese industry.

Solid state fermentation (SSF) technique is particularly suitable for the production of fungal enzymes, due to their potential advantages in manufacturing products such as high yields, low energy consumption, low environmental impact of the process, differential expression of metabolites and requirement of less expensive technology and skill. However, the scale-up and optimization of SSF processes that is necessary for commercial production is complex and demand intensive research (Gelmi *et al.,* 2000).

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 rennet is their high thermal stability which allows them to extend their action on milk proteins after coagulation. Although after cheese making only 0 to 15% of the rennet activity added to milk is retained in the curd (Sousa *et al*., 2001), the mentioned characteristics of commercial fungal rennet 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,

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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. Aspartic proteases produced from, three species, namely *Rhizomucor miehei*, *Rhizomucor pusillus* and *Cryphonectria parasitica* have been established for large scale production and have, like calf rennet, less heat stability than those from other fungi (Jacob *et al*., 2011).

The aim of the present study was to screen fungal strains for milk clotting enzyme production under solid state fermentation (SSF). The growth conditions of the promising strain in solid state fermentation were investigated to explore the production of milk clotting enzyme and to obtain a better understanding on the biology of the tested organism. This stage was followed by large scale production of the enzyme under solid state fermentation using aluminum trays. Also, the biochemical properties of the produced enzyme were investigated.

MATERIALS AND METHODS

2.1 Microorganisms and Maintenance Condition:

Fungal cultures used in this study were *Rhizomucor miehei* NRRL 2034, *Rhizomucor miehei* NRRL 3169, *Rhizopus oligosporus* NRRL 2710 and *Rhizopus oligosporus* NRRL 2549. They were obtained from Northern Regional Research Laboratory, Peoria Illinois, USA. These cultures were periodically subcultured and maintained on potato dextrose agar medium.

2.2 Inoculum Preparation:

The inoculum for SSF was prepared by scrapping the surface of five days old cultures on PDA medium with sterile distilled water. The spore suspension was used as inoculum.

2.3 SSF (Solid State Fermentation):

Various industrial residues such as cotton seed meal, wheat bran, rice hull, fodder yeast, orange peels, potato peels, rice straw, pea nut peels, lenin meal, sesame meal, soybean meal and *Nigilla sativa* meal were collected from local market and evaluated for their potential as substrates in solid state fermentation for milk clotting enzyme production. All the industrial residues were dried at 60^{o}C for one hour, grinded and used for milk clotting enzyme production. Industrial by-products distributed separately in Petri dishes, moistened with distilled water or mineral salt solution (Thakur *et al.*, 1990) and autoclaved for 30 min then inoculated with about 1.8 ×10^{8} CFU/g of spore suspention and incubated at 40ºC.

2.4 Optimization of Cultural Parameters:

Using solid state fermentation, milk clotting protease production was studied at different moisture content (10-80% v/w), different temperatures (30-50ºC), inoculum sizes (1.8×10^{8}- 9×10^{8} CFU/g), various nitrogen sources (skim milk, casein, gelatin, casein hydrolysate and whey powder), different moistening agents (corn steep liquor, whey permeate, cane molasses, distilled water, tap water, mineral solution, 0.1N HCl and 0.2N HCl), incubation time (1-7 days) and wheat bran particle size (>300 to 75 µm).

2.5 Enzyme Extraction:

After 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 2 h at 30ºC. The filtrate obtained was centrifuged at 4000 rpm for 10 min. The supernatant was used as crude enzyme source for milk clotting activity.

2.6 Milk Clotting Activity (MCA):

MCA was determined according to the method of Greenberg (1957). Two milliliter of skim milk solution (12% in 0.01 M $CaCl_2$) was incubated at 35ºC. Then 0.2 ml of enzyme was added and the time for curd formation was recorded. Three replica were examined and an average of these was used in the calculation of the enzyme units. 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 1 ml of skim milk in 40 min at 35^{o}C.

2.7 Protease Activity (PA):

Protease activity was measured using casein as a substrate. One ml of 1% casein in phosphate buffer (pH 7) and 0.5 ml of enzyme were incubated at 35°C for 30 min. The reaction was terminated by addition of 2 ml of 15% trichloroacetic acid solution and was centrifuged at 6000 ×*g* for 10 min. To 1.0 ml of supernatant, 2.5 ml of 0.5 mol/l of sodium hydroxide and 0.5 ml of Folin reagent were added, followed by 30 min incubation at room

temperature. Absorbance was measured at 650 nm. One unit of protease activity was defined as the amount of enzyme, which released 1 µg of amino acid equivalent to tyrosine under the assay conditions.

2.8 Protein Determination:

Enzyme protein content was estimated according to Ohnistti and Barr (1978) using bovine serum albumin as standard.

2.9 Large Scale Production of Milk Clotting Enzyme:

The standardized wheat bran medium with 50% moisture content was evenly distributed in aluminum foil trays (20 × 25 ×5 cm^3) to different depths (0.5-4 cm). The trays were sterilized in the autoclave for 30 minutes. The medium was inoculated with the actively growing starter cultures of *Rhizomucor miehei* and cultivation was carried out at 40ºC. Ten trays of each run were loaded at a time for the enzyme production under the standard conditions.

2.10 Biochemical Properties of the Enzyme:

Temperature profile was determined at different temperatures (30-70°C). The thermal stability of the enzyme was ascertained by measuring the activity of the residual enzyme exposed at various temperatures (30-70°C) for ten minutes. Effect of pH values on the activity of the enzyme was studied at pH range 3.5-7 by using appropriate buffer solutions. Effect of calcium chloride (0.002-0.2M) and sodium chloride (3-12%) concentrations on MCA of the enzyme were tested. The effect of the substrate concentration (3-18% skim milk) was investigated.

RESULTS AND DISCUSSION

Screening of Fungal Strains:

Aspartate proteases play an important role in the manufacture of cheese in dairy industry. Increased demand for cheese, coupled with ethical and low availability of rennet has led to replacement of calf rennet by microbial milk clotting enzymes. However, these microbial milk coagulants are associated with non-specific and heat-stable proteases which lead to the development of bitterness in cheese after storage and a poor yield. Search is on for the low-cost methods of producing enzymes that are completely inactivated at the normal pasteurization temperatures and contain very low levels of contaminating proteases.

In this study four fungal strains were screened for their abilities to produce milk clotting enzyme under solid state fermentation conditions. As shown in Table 1, *Rhizomucor miehei* NRRL 2034 produced the highest enzyme productivity reaching 35500 SU/g fermented culture after four days of incubation under solid state fermentation with high MCA to PA ratio (11.9:1). Thus, *Rhizomucor miehei NRRL* 2034 was chosen alongside this study.

The aspartic protease produced by *Rhizomucor miehei* consists of a single polypeptide chain with a high similarity to calf rennet in its three dimensional structure (Chitpinityol and Crabbe 1998) and its properties (Jacob *et al*., 2011).

Table 1: Screening of fungal strains grown under SSF conditions for milk clotting enzyme production.

Fungal Strain	Final weight (g)	Final pH	MCA (SU/g)	MCA/PA
After 3days				
Rhizomucor miehei NRRL 2034	9.5	6.5	31750	10.9
Rhizomucor miehei NRRL 3169	9.4	5.8	30000	8.9
Rhizopus oligosporus NRRL 2710	8.4	4.3	12650	10
Rhizopus oligosporus NRRL 2549	7.6	4.0	5450	5
After 4 days				
Rhizomucor miehei NRRL 2034	9.5	6.2	35500	11.9
Rhizomucor miehei NRRL 3169	10.1	5.7	32250	8.4
Rhizopus oligosporus NRRL 2710	8.5	4.7	15000	11.9
Rhizopus oligosporus NRRL 2549	8.0	4.5	6665	5.1

Effect of Industrial by-Products As Substrates on Enzyme Productivity:

The selection of an ideal industrial residue for enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate material, and thus may involve screening of several industrial residues (Pandey *et al*., 2000).

Twelve industrial by-products were tested separately as a nutrient substrate for *Rhizomucor miehei* NRRL 2034 under SSF. The obtained results showed that wheat bran yielded the highest MCA as shown in Table 2. These results are in agreement with several reports describing wheat bran as a potent substrate for milk clotting enzyme production by *Aspergillus oryzae* MTCC 5341 (Vishwanatha *et al*., 2010), *Rhizomucor* species (Preetha and Boopathy, 1994) , *Rhizomucor miehei* (Thakur *et al*, 1990; Preetha and Boopathy, 1997) and *Rhizomucor pusillus* (Nouani, *et al*, 2011).

Table 2: Effect of different industrial by-products on milk clotting enzyme production of *Rhizomucor miehei* NRRL2034 under SSF conditions.

By-product	Final weight (g)	Final pH	MCA (SU/g)	MCA/PA
Cotton seed meal	10.0	6.3	7000	3.5
Wheat bran	9.8	5.4	33350	10.9
Rice hull	8.5	5.4	3600	3.8
Fodder yeast	9.2	5.6	4400	2.7
Orange peels	9.2	4.2	0	-
Potato peels	8.3	4.7	0	-
Rice straw	6.8	5.6	0	-
Pea nut peels	7.7	5.1	4600	1.5
Lenin meal	9.4	5.7	3900	1.1
Sesame meal	9.6	5.8	15000	4.5
Soy bean meal	10.0	5.8	18450	3.6
Nigella sativa meal	9.2	6.0	17150	5.2

Although orange peels, potato peels, and rice straw enhanced the vegetative growth of the tested organism, failed to induce formation of milk clotting enzyme as no MCA was obtained up to 120 minutes of reaction time. This inhibition perhaps related to the presence of some milk clotting enzyme inhibitory substances in these by-products media.

In another experiment, mixtures of wheat bran with fodder yeast, cotton seed meal, sesame meal, soy bean meal, *Nigella sativa* meal and Lenin seed meal separately in the ratio of 1: 1 were carried out. The growth of the tested organism on these mixtures showed less enzyme yield than cultivation on wheat bran alone (data not shown). Hence, based on these observations, wheat bran was selected and used as a substrate for further optimization studies.

Influence of Moisture Content on Enzyme Production:

Among the several factors that are important for microbial growth and enzyme production under solid state fermentation using a particular substrate, moisture level (content)/ water activity which is one of the most critical factor. Because, SSF process are different from submerged fermentation culturing, since microbial growth and product formation occurs at or near the surface particle having low moisture content (Preetha and Boopathy, 1994; Pandey 2000).

In this study, the maximum enzyme production was observed with 50% moisture content (33350 SU/g fermented culture) as shown inTable 3. At lower and higher initial moisture levels, the metabolic activities of the culture and consequently product synthesis was variously affected. This could be explained by the fact that lower moisture levels lead to reduced solubility of the nutrients in the solid substrates, a lower degree of substrates swelling and higher water tension. Similarly, higher moisture content were reported to cause decreased porosity, loss of particulate structure, development of stickiness, reduction in gas volume, decreased gas exchange and enhanced formation of aerial mycelium (Pandey 2000). Similar findings have been reported by other workers (Thakur *et al*., 1990, Sathya *et al*., 2009, Jacob *et al*., 2011).

Table 3: Effect of moisture content on milk clotting enzyme produced by *Rhizomucor miehei* NRRL 2034 grown under SSF conditions.

Moisture content (%)	Final weight (g)	Final pH	MCA (SU/g)	MCA/PA
10	5.1	5.0	9300	4.8
20	5.0	4.7	12500	3.9
30	5.6	4.4	21500	7.2
40	5.6	4.2	27500	11
50	7.9	4.4	33350	10.9
60	5.2	4.5	32700	10.5
70	6.4	4.3	29050	10.7
80	8.5	4.5	23650	10.3

Effect of Incubation Temperature:

It is known that temperature is one of the most critical parameter that has to be controlled in bioprocess. *Rhizomucor miehei* under SSF exhibited the highest enzyme production at 40 ^{o}C (34200 SU/g fermented culture) as shown in Table 4. Increasing or lowering the cultivation temperature resulted in the reduction of

enzyme production. In various studies, it was observed that the production of milk clotting enzyme by microorganism was affected by cultivation temperature (Holker and Lenz 2005, Thakur *et al*., 1990; Sathya *et al*., 2009). Optimum temperature for growth and milk clotting enzyme production by *Rhizomucor miehei* was 42°C and 40°C as reported by Thakur *et al*., 1990 and Vaysari *et al.*, 2002, respectively.

Table 4: Effect of incubation temperature on milk clotting enzyme produced by *Rhizomucor miehei* NRRL 2034 grown under SSF conditions.

Incubation temperature (°C)	Final weight (g)	Final pH	MCA (SU/g)	MCA/PA
30	10.6	6.3	22850	8.4
40	6.4	4.3	34200	11
50	6.4	4.5	25250	10

Influence of Inoculum Size:

Size of inoculum is an important biological factor, which determines biomass production in the fermentation process. The results presented in Table 5 showed gradual decrease in milk clotting enzyme production with increasing the inoculum size. Maximum enzyme production (37500 SU/g fermented culture) was obtained when SSF medium was inoculated with 1.8×10^8 CFU/g. The decrease in milk clotting enzyme with larger inoculum size could be due to the shortage of the nutrients available for the larger biomass and faster growth of the culture. Hence, a balance between the proliferating biomass and available material is essential to yield maximum enzyme production as reported by Hesseltine *et al*. (1976). It was reported by Sathya *et al* (2009) that 30% inoculum size was an optimal level for milk clotting enzyme production by *Rhizomucor circinelloides* under SSF after which enzyme yield was reduced.

Table 5: Effect of inoculum size on milk clotting enzyme produced by *Rhizomucor miehei* NRRL 2034 grown under SSF conditions.

Inoculum size ($\times 10^8$ CFU /g)	Final weight (g)	Final pH	MCA (SU/g)	MCA/PA
1.8	9.4	5.9	37500	11.1
3.6	10.8	6.1	28550	8.8
5.4	12.0	6.1	26100	8.7
7.2	11.4	6.0	10900	4.5
9	12.0	6.1	5000	2

Effect of Different Nitrogen Sources on Enzyme Production:

Among the various nitrogen sources tested, whey powder at 2% was found to be the best nitrogen source for the enzyme production (48600 SU/g fermented culture) followed by skim milk at 3% (42850 SU/g) then casein at 2% (39200 SU/g) as shown in Table 6. It is interesting to note that, no reports available on the production of milk clotting enzyme using whey as a nitrogen source which represents an inexpensive promising source of nitrogen. On the other hand, casein hydrolysate and gelatin clearly inhibited enzyme biosynthesis reaching to the minimum enzyme activity at 10% final concentration. da Silveira *et al* (2005) and Yegin *et al*. (2010) reported that casein inhanced milk clotting enzyme production by *Rhizomucor miehei* and *Rhizomucor mucedo*, respectively. Thakur *et al*. (1990) and Preetha and Boopathy (1994) studied the influence of the skim milk inclusion in solid medium and observed that increasing the concentration more than 2% resulted in an increase in the coagulation activity.

Table 6: Effect of different nitrogen sources on milk clotting enzyme produced by *Rhizomucor miehei* NRRL 2034 grown under SSF conditions.

Nitrogen Source	Final weight (g)	Final pH	MCA (SU/g)	MCA/PA
None	5.6	5.5	34200	11
Skim milk				
1%	4.6	5.5	38100	8.1
2%	5.1	5.8	38450	8.5
3%	6.2	6.3	42850	8.9
4%	4.9	6.4	31250	6.2
5%	4.2	6.1	30750	5.3
10%	4.6	6.5	28550	4.5
Gelatin				
1%	6.8	6.1	20000	3.8
2%	6.5	6.0	14350	2.1
3%	6.4	6.1	13000	1.7
4%	6.5	6.2	11650	1.5
5%	6.9	6.3	11750	1.5
10%	6.9	6.3	9600	1.3
Casein				
1%	4.1	7.3	35700	7.2
2%	6.8	7.2	39200	9.4

3%	5.0	7.3	37500	10.9
4%	4.3	7.2	34500	6.8
5%	4.3	7.0	26800	5
10%	4.8	6.9	25850	5.6
Casein hydrolysate				
1%	5.6	6.3	25000	7
2%	6.2	6.1	25600	7.8
3%	6.5	6.0	9250	2.9
4%	6.6	5.9	8750	2.9
5%	6.7	5.7	3100	6.5
10%	6.9	5.8	0	-
Whey				
1%	5.8	6.4	39250	11.3
2%	5.0	6.5	48600	11.6
3%	6.5	6.6	42850	8.7
4%	6.5	6.7	39200	8.7
5%	7.6	6.8	37500	8.8
10%	7.2	6.9	30300	8.8

Da Lima *et al* 2008 found that *Rhizomucor miehei* could use deproteinized whey as carbon source and enhanced the milk clotting enzyme production. They reported that this increment in milk clotting enzyme was due to the ability of this fungus to break the lactose of deproteinized whey to glucose through the galactosidase enzyme.

Effect of Different Moistening Agents on Enzyme Productivity:

In this study wheat bran enhanced milk clotting enzyme productivity with the most examined moistening agents except for using 0.1N HCl and 0.2 N HCl as shown in Table 7. The highest milk clotting activity was achieved by using mineral solution as a moistening agent for wheat bran reaching 56200 SU/g fermented culture followed by tap water (53500 SU/g fermented culture).

It is known that metal ions are required in the fermentation media for optimum production of proteases. However, the requirements of specific metal ions depend on the sources of enzymes. It was found that the addition of Mg^2, Ca^2, and Cu^2 ions increased milk clotting enzyme production by *Rhizomucor circinelloides* as reported by Sathya *et al*. (2009).

Effect of Incubation Period on Enzyme Productivity:

Result of this study showed that the milk clotting enzyme production was increased with incubation time up to three days (61550 SU/g) as shown in Table 8. Further incubation showed reduction in the enzyme production. The reduction in enzyme yield after optimum period was probably due to depletion of nutrients available to microorganisms. Similar findings have been reported by other workers (Thakur *et al*., 1990; da Silveira *et al*., 2005; Sathya *et al*., 2009).

Effect of Wheat Bran Particle Size:

An increment in MCA (16%) was achieved with the fraction of > 300 µm of wheat bran as shown in Table 9. On the other hand, 75 µm fraction failed to produce detectable coagulation activity. Large fractions of wheat bran in SSF may increase the aeration, increase the gas exchange and decrease the stickiness of the medium.

Large Scale Studies in Trays:

A large scale production of the enzyme was carried out in trays with different medium thickness applying optimum culture conditions obtained in small scale study. Maximum activity was obtained after 72 h of fermentation with the highest activity of 63550 SU/g fermented culture in trays with 0.5 cm thickness as shown in Table 10.

Thakur *et al*. 1990 could produce *Rhizomucor miehei* with high MCA (45262 SU/g fermented culture) in wheat bran medium under SSF. Vishwanatha *et al*. (2010) reported that milk clotting enzyme produced by *Aspergillus oryzae* under SSF was 40000 SU/g fermented culture. The milk clotting enzyme activities obtained in present study with *Rhizomucor miehei* under SSF are promising from the point of view of the development of a commercial process. The yield obtained is higher than the highest milk clotting activities reported so far under SSF. Further a ratio of milk clotting to proteolytic activities of 10:1 has been achieved in the crude extract which is comparable to commercial rennet substitutes in the market.

Characterization of Milk Clotting Enzyme:

Effect of Temperature on Enzyme Activity and Stability:

The obtained results indicated that MCA increased progressively with increasing the incubation temperature reaching the highest MCA value at 60^{o}C as shown in Figure 1. At higher incubation temperatures, MCA

decreased. Milk clotting enzyme was stable up to 50°C (Figure 2). However, heating the enzyme at 60°C for 10 minutes drastically reduced MCA to 40 % and PA to 63% whereas total denaturation of the enzyme was evident at higher temperatures.

Table 7: Effect of different moistening agents on milk clotting enzyme production by *Rhizomucor miehei* NRRL 2034 grown under SSFconditions.

Moistening agent	Final weight (g)	Final pH	MCA (SU/g)	MCA/PA
2% corn steep liquor	9.9	4.6	38300	10.8
Permeate	11.4	4.4	47450	10.3
Cane molasses (2%)	10.9	4.3	35550	11.5
Distilled water	9.2	4.6	48600	11.7
Tap water	9.1	4.3	53500	11.6
Mineral solution	9.9	4.4	56200	11.7
0.1 N HCL	9.7	3.7	18000	10.9
0.2 N HCL	8.8	2.9	5550	4.2

Table 8: Effect of incubation period on milk clotting production by *Rhizomucor miehei* NRRL 2034 grown under SSF conditions.

Incubation period (days)	Final weight (g)	Final pH	MCA (SU/g)	MCA/PA
1	9.2	5.1	48550	10.9
2	10.2	5.4	48250	11.3
3	8.3	5.4	61550	14.0
4	8.5	5.5	60600	14.2
7	5.0	6.3	21400	8.5

Table 9: Effect of wheat bran particle size on milk clotting enzyme produced by *Rhizomucor miehei* NRRL 2034 grown on wheat bran medium under SSF conditions.

Paticle size (µ m)	pH at Harvest	MCA SU/g	MCA/PA
Control	6.1	61550	11
> 300	6.3	71400	11.5
300	6.5	38100	8.3
150	5.9	28550	6.8
75	5.5	3750	1.4

Table 10: Large scale production of milk clotting enzyme under SSF conditions.

wheat bran weight (g)/thichness	Final weight (g)	Final pH	MCA (SU/g)	MCA/PA
125/0.5	265	5.6	63550	10
250/1	600	5.6	41550	9.1
375/1.5	700	6.0	30500	8.4
500/2	950	6.2	17750	7.5
750/3	1200	6.2	2542	2.2

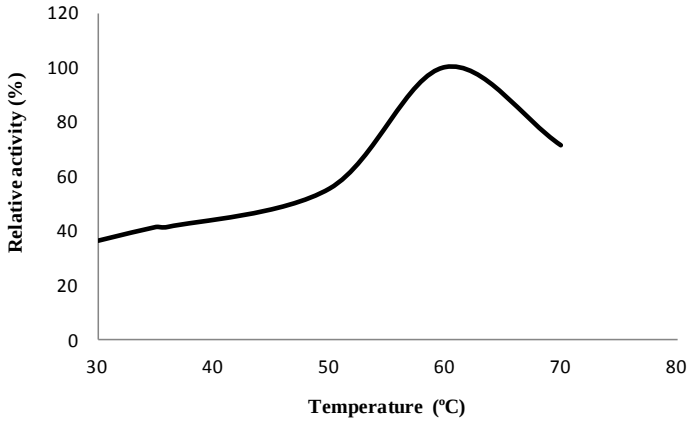

Fig. 1: Optimum temperature of milk clotting enzyme produced under SSF.

It was reported that, milk clotting activity of *Rhizomucor pusillus* was the best at 50°C (Nouani *et al*. 2009). Milk clotting enzyme from *Aspergillus oryzae* had an optimum temperature of 55 °C and had a midpoint for thermal inactivation of 49°C (Vishwanatha *et al*., 2010). This coincides with the midpoint of thermal inactivation for calf rennet (Martin *et al*. 1980). Thermolability is another preferred quality for microbial milk clotting enzymes as undesired proteolysis of proteins, beyond normal pasteurization temperature, can be avoided.

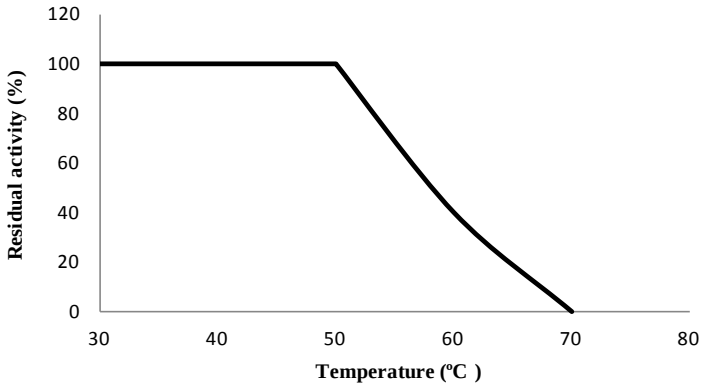

Fig. 2: Thermal stability of milk clotting enzyme produced under SSF.

Optimum pH:

Optimum pH for the maximum MCA was found to be pH 5 (Figure 3). Further acidification showed acidic clotting of milk. At pH values higher than pH 5 MCA decreased to reach 40% at pH 7.

It was reported that, pH has a profound impact on milk clotting activity by rennet and pH value less than 5 cause curdling of the milk. Microbial rennet is reported to have an optimal pH (5.5–7). The optimum pH of calf rennet about 6–6.3 as reported by Okigbo *et al*. 1985. The optimum pH for milk clotting enzyme of *Aspergillus oryzae* was pH 6.3 (Vishwanatha *et al*., 2010). It was reported that the optimum milk clotting activity was at pH 5.6 and 6.0 for *Rhizomucor miehei* and *Rhizomucor pusillus,* respectively (Jacob *et al*., 2011).

Effect of Calcium Chloride:

The results illustrated in Figure 4 showed that the coagulation activity of the enzyme was greatly stimulated with addition of $CaCl_2$ up to 0.05M. However, higher concentrations resulted in a marked decrease in MCA which was reduced to 37.5 % at 0.2M $CaCl_2$.

As reported earlier, calcium ions in the reaction mixture decrease the rennet clotting time (Balcones *et al*. 1996; Montilla *et al*. 1995, Kumar *et al*. 2005). Addition of calcium also reduces pH of the milk solution thereby hastening protein aggregation (Gastaldi *et al*. 1994). Curd firmness, gel strength, aggregation rate, and adhesiveness are improved with the addition of calcium ion (Solorza and Bell 1998; Cavalcanti *et al*. 2004).

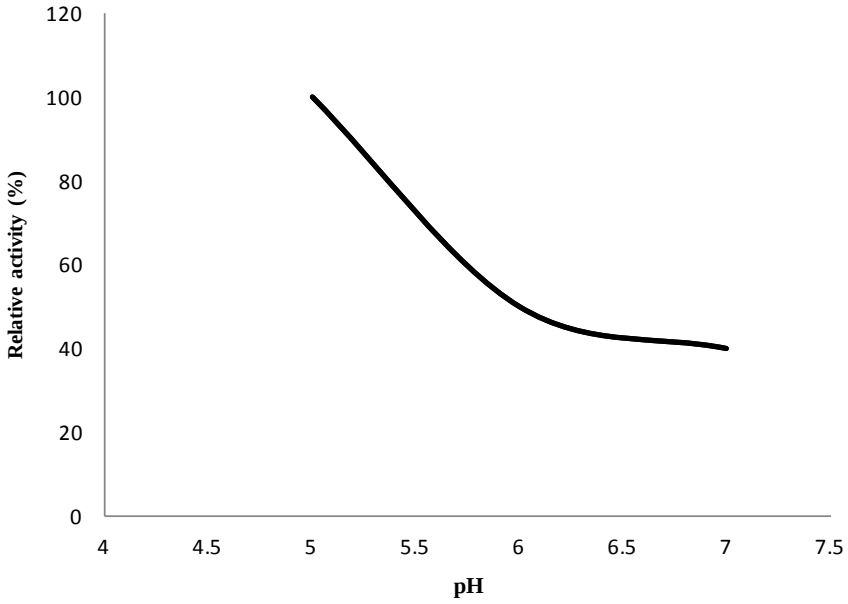

Fig. 3: Optimum pH of milk clotting enzyme produced under SSF.

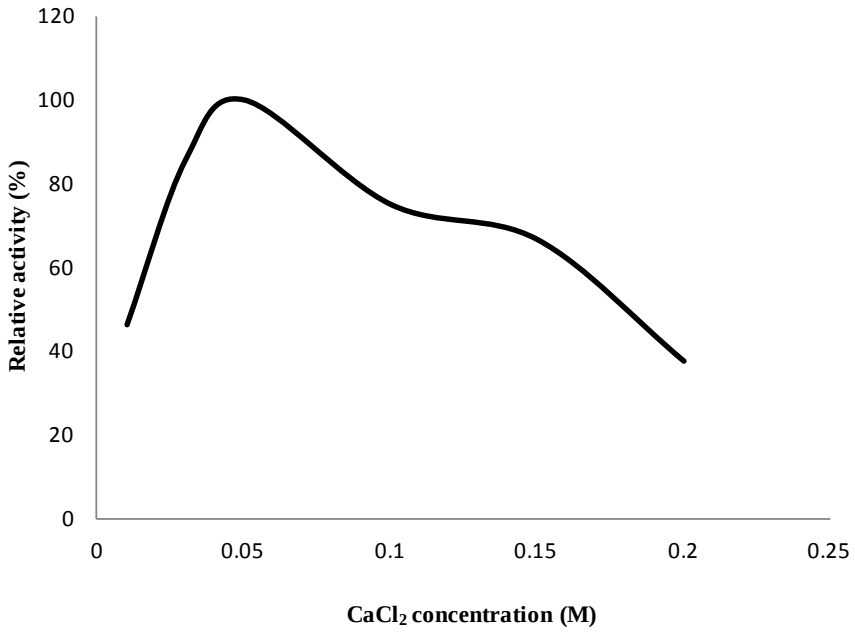

Fig. 4: Effect of $CaCl_2$ concentration on MCA.

Effect of Sodium Chloride:

The obtained results show a progressive inhibition of the MCA with the increase of the concentration of sodium chloride as shown in Figure 5.

Milk is sometimes salted with sodium chloride for protection against spoilage by various microorganisms. The amount of salt required to reduce water activity to prevent microbial growth is 4-5%. The sensitivity of milk clotting enzyme from various sources to sodium chloride is not the same. Bovine pepsin appears less sensitive to sodium chloride than calf rennet, particularly at high salt concentrations (Ramet 2001).

Effect of Skim Milk Concentration:

It is clear that the highest MCA was obtained at 9% skim milk as shown in Figure 6. At more or less skim milk concentrations than this level, MCA gradually decreased.

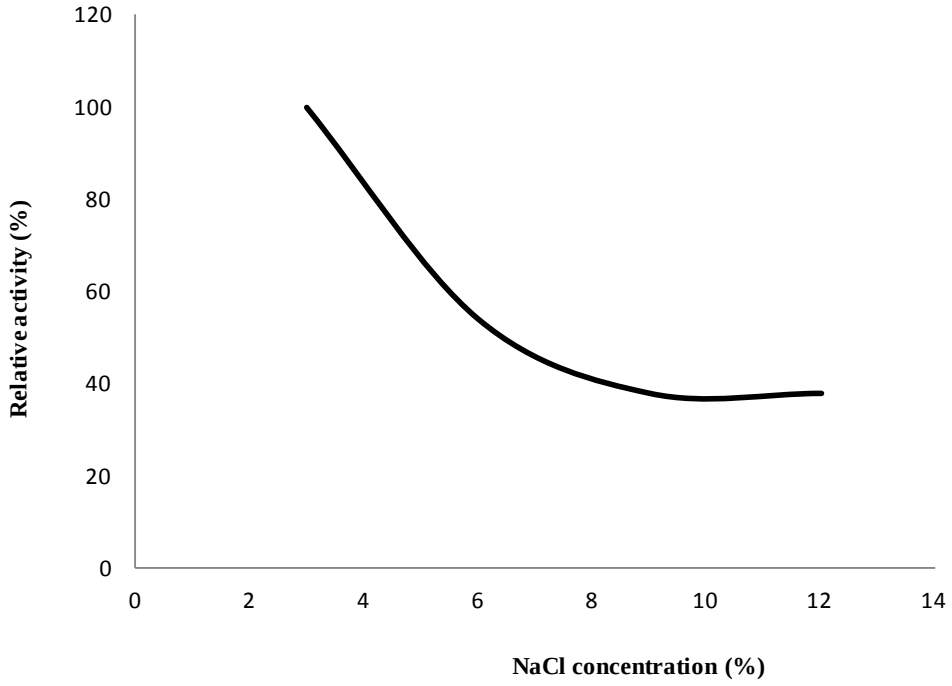

Fig. 5: Effect of NaCl concentration.

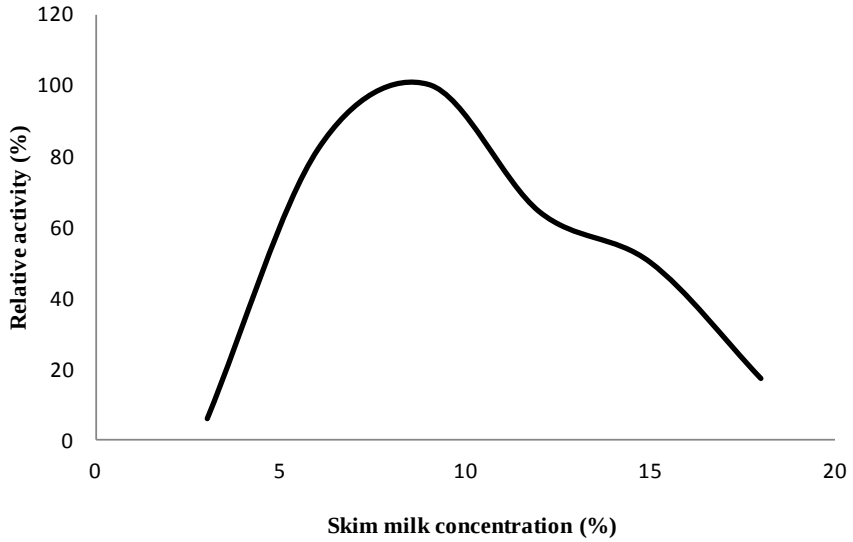

Fig. 6: Effect of skim milk concentration.

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