

Optimization of Fungal Rennet Production by local isolate of *Rhizomucor nainitalensis* Under Solid Substrate Fermentation system

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Abstract: In this study the production of substitute rennet by a newly isolated thermophilic fungus, *Rhizomucor nainitalensis*, using optimized solid substrate fermentation system have been explored. The results revealed that enzyme activity is at its highest level at 40°C, 50 % humidity, inoculation volume of 10³ spore /gr wheat bran, and exoenzyme biosynthesis induction by casein at a rate of 1.5 %. Enzyme activity reached its highest (8400 SU/gr wheat bran) after 110 hours of incubation under the stated conditions. Precipitated enzyme, by addition of ammonium sulfate with milk coagulating activity of 115000 SU/gr enzyme powder, was free of toxin and microorganisms, with market's favorable aroma.

Keywords- *Rhizomucor*, Solid Substrate Fermentation, rennin, fungal proteases

I. Introduction

Cheese contains the vital amino acids and calcium and is considered an important food as human digestive system absorbs cheeses' protein more easily than of meat's protein. Cheese contains considerable amount of calcium to phosphor ratio and vitamins A, D [1,2]. In addition to starter cultures, Cheese production requires substances, which cause curdling of proteins. These substances could be enzymes, lactic acid producing bacteria or even acids and acidogenics [3,4]. The highest quality cheese, though, is made by using enzymes. Such cheese can be given different flavors and has a higher content of calcium as well. When curdling of proteins is achieved through enzymes, the result is a new protein lattice called a curd or clot [5,6]. Until early 70's, the needed enzymes were extracted from the inner mucosa of the fourth stomach chamber (the abomasum) of slaughtered young, unweaned calves.

The extract is called rennet and its purified and active ingredient is called Rennin or Chymosin. According to the Enzyme Nomenclature by International Union Of Biochemistry And Molecular Biology (IUBMB) Rennin, E.C 3.4.23.4, is an aspartic Proteinase that is a member of the Hydrolase group [7]. These enzymes can cause milk proteins to curdle by selectively breaking up peptide bonds between methionine 106 and phenylalanine 105 amino acids within the Kappa casein chain [8]. Rennet provides higher content of dry substance in produced cheese and the yield ratio of cheese to the consumed milk is higher so the enzymatic milk coagulation method is economical compare to other methods since [9].

Production of traditional rennet has been a problematic issue for some time now, and one of the most important reasons is the high price of meat and unwillingness of breeders to slaughter infant calves for rennin. Moreover, the high prices of produced calf rennet, ethical and religious barriers are some other causes to limit the production of traditional rennet for the past decades. Limited sources for calf rennet on one hand and ascending consumption and demand for cheese on other hand is another reason drawing the attention of scientists to find rennet substitutes before world faces its actual shortage. These efforts have greatly expanded in recent decades [3].

Many enzymes are capable of hydrolyzing kappa-casein and causing the milk to curdle however, the majority cannot be suitable candidates for dairy industries because of different reasons. To be useful as a substitute of natural rennet, a substance's proteolytic activity should be similar to the rennet extracted from calves' abomasum [7,10]. It should not wreak unwanted odors or cause unwanted flavor in the final product, cheese. It should not alter organoleptic characteristics of cheese and should be able to curdle milk at the same conditions (including Calcium ion concentration, pH and temperature) as natural rennet. It should not cause unwanted bacterial growth, should not be poisonous, or result in a poisonous final product. In addition, it should not cause unwanted antibiotic activity, which may hinder the growth of lactic bacteria, its production should not be too costly and its use should not be limited to specific production situations [11-13].

Earlier research was focused on substitutes of plant origin (*Carica papaya*, *Ficus carica*, *withania cocagulans*) [14,15] or of animal origin (poultry pepsin or omnivores' pepsin) [5,16]. Later, focus was shifted on

studies on the rennet substitutes with microbial origins. With regards to the aforementioned selection criteria, the worldwide studies on substitute rennet were limited to few classes of bacteria and fungi which later were narrowed down to three species; two species of fungi, *Rhizomucor miehei* [17,18] and *Mucor pusillus* [19], found in soil and one species of yeast (*Endothia parasitica*) a parasite of oak tree [20]. Today, almost all produced microbial rennets in the world are produced using these three microorganisms and are sold under different commercial names including Fromase, Meito MR, Noury rennet, Marzyme, Suparen, and Novo rennet.

In general, three different systems of fermentation are used for biosynthesis of enzymes, solid substrate (state) fermentation (SSF), liquid substrate fermentation (LSF), and submerged fermentation (SF) [21,22]. Among these, SF and SSF system have been used widely because of their higher yield [23]. Submerged fermentation systems are those in which fermentation is achieved in a liquid medium and are used to produce many metabolites and since fungi are aerobic microorganisms, production of enzymes would require shaker-equipped incubator or fermenters with aeration system. Unlike the submerged fermentation system, in solid substrate fermentation system, nutrients are not in the form of liquid suspension and there are no “free” and “solved” substrates. The term solid substrate fermentation was first used to describe a process through which agricultural food products were enriched and fortified for improved digestibility. This method does not require highly advanced technology and compare to its high yield, the size of reactor is small. Product’s initial concentration is already high, increasing its concentration is not too costly, and so this method is economical compare to other methods. These advantages have made this system favorable in production of exoenzyme, mono-cell proteins, fermented foodstuff, toxins, organic acids, and some industrial chemicals. Invertase, Cellulose, Lactase, Aminoglycosides, Pectinase and fungal proteases, including those used as substitute rennet, can be biosynthesized through this method. SSF fermenters need much less energy than those of other methods. They also require smaller space than shaker-equipped cylindrical reactors. These characteristics make SSF ideal for use in developing as well as industrial countries [24,25].

In biotechnological processes, natural raw material, or recyclable waste material such as lignin, bran, wheat flour, rice flour, cotton, yeast extract, soy powder, beet molasses, starch, and cellulose are widely used as substrate. Among these, wheat bran has specially been recognized as a good choice for industrial production of enzymes. Wheat bran is comprised of 69% carbohydrate, 15% protein, phosphate, calcium, iron, sodium, copper, chlorine, lipids and vitamins and is exclusively suitable for use in production of fungal enzymes [26,27].

During recent years rennin-like enzymes after bacterial proteases used in tannery and bakery industries, have constituted a big share of the \$2.1 billion dollars to the industrial enzymes market, worldwide [28]. Among rennet enzymes, microbial rennets make up a big part of the sales. In the most of third world and developing countries, 100 % of the consumed rennets used in dairy industries is imported from industrialized countries. In this paper we are presenting the results of the trial production of microfungus rennet by locally isolated *Rhizomucor nainitalensis* in our laboratory, using solid substrate fermentation system [29]. This may open up a path in industrial scale production of the rennet enzyme in near future.

II. Material and Method

- 2.1. **Microorganism:** we collected local isolates of *Rhizomucor nainitalensis* and stored the isolates on potato dextrose agar (PDA) slants which were used in this study. They were kept at 4°C after undergoing 10 days of incubation at 35°C for inducing sporulation. To keep the microorganism active, the isolates were transferred to PDA slants at one-month intervals.
- 2.2. **Culture medium:** production of aspartic proteases were studied by the use of six types of solid complex media. Wheat bran was chosen as the main carbon source and was the base medium of all the six. Different substances or a combination of those were added, in a percentage of 10 %, to the base medium to study the effect of different carbon or nitrogen source on the production of the enzyme. The added substances were: 1-Wheat flour, 2-Sorghum flour, 3-Peanut flour, 4-Coconut powder, 5-Rice flour, 6- No addition (Blank). Incubation parameters were set at 40 % humidity temperature at 35°C and incubation time of 180 hours.
- 2.3. **Inoculation of the culture medium:** cultures of *Rhizomucor* were kept for 10 days on PDA slant and were used for inoculation of the solid substrate, wheat bran. Concentration of spore suspension was determined by counting on an advanced Neubauer Counting chamber and then was used for the inoculation of the culture medium.
- 2.4. **Enzyme source:** Rectangular stainless steel trays 38cm x 26cm x 8cm with lid (so called Instrument-Tray style), holding the culture medium and inoculated with spore suspension were placed in damp incubator for 180 hours. Trays were taken out of the incubator and distilled water, 4°C, was added to stop the fermentation. Enzyme solution was extracted by filtering through Whatman paper (No.1) and separating the bran. The filtrate was kept in 2°C until the time enzyme assays were conducted.
- 2.5. **Assay of Enzyme Activity:** Curdling potential of the enzyme was measured using Arima (1972) method and recorded in Soxhlet units. One Soxhlet is the amount of enzyme which can cause 1 ml of suspension

containing 0.1% of fat free skimmed milk and 0.1 m of calcium chloride to curdle at 35°C in 40 minutes. To measure the curdling potential, extracted enzyme was added to substrate suspension (1:10) at the aforementioned conditions. The time taken for the milk to curdle at 35°C was recorded and enzyme activity was calculated with the formula below:

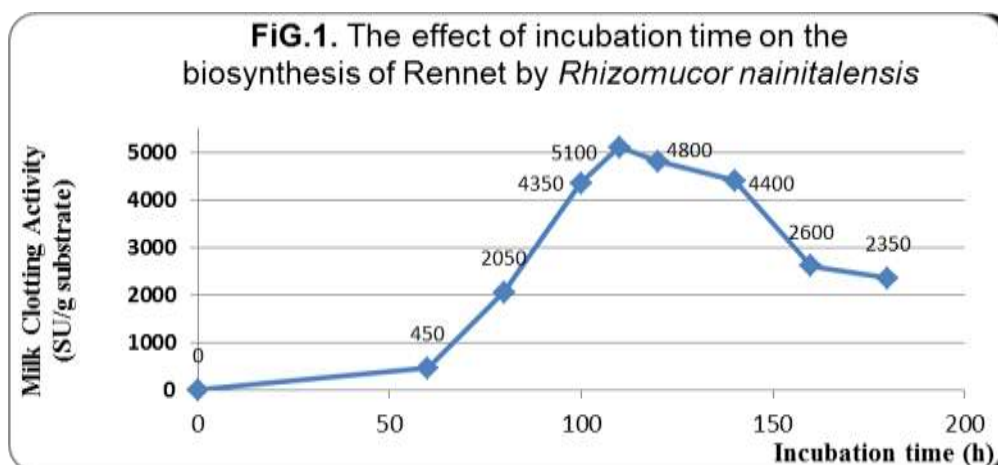
$$U = \frac{M(m.l)}{E(m.l)} \times \frac{35^\circ}{t(^{\circ})} \times \frac{2400}{T(sec)}$$

Where U: curdling potential of the enzyme in Soxhlet, M: volume of the substrate, E: volume of the enzyme, t: Temperature of the substrate-enzyme reaction, T: Time taken for curdle to form.

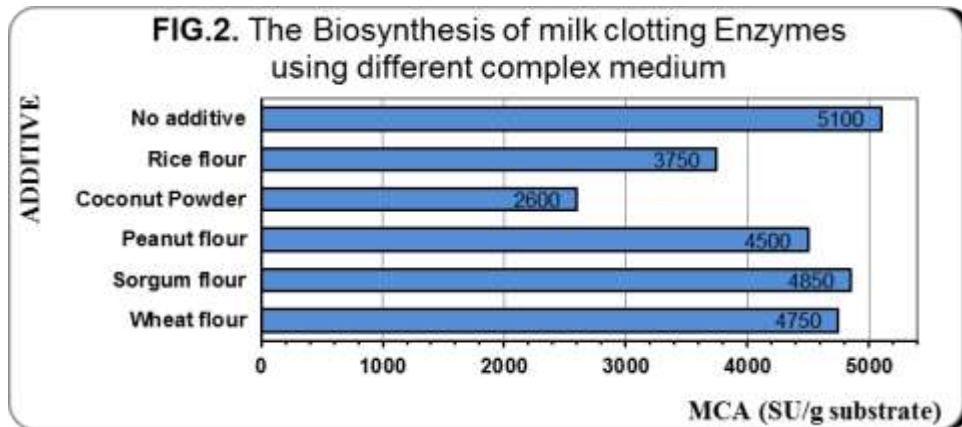
2.6. **Protease Activity (PA) of enzymes:** PA of each protease can be measured through several methods including methods of Bailey (1988), Anson (1938), and Kunitz (1947). These methods use skimmed milk, hemoglobin, and casein as substrate [30-32]. We used Kunitz method with a combination of casein and hemoglobin as substrate. The proteolytic activity was expressed in units per ml (U/ml). Briefly, 1ml of enzyme solution was added to 5ml of substrate solution containing 1.2% of casein solution in 0.05 Mole phosphate buffer (pH 6.0). The mixture was incubated at 35°C for 10min then 5ml of 0.44 Mole Tri-Chloroacetic Acid (TCA) was added to inhibit the reaction. After filtration, 2ml of the filtrate was added to 5 ml of NaOH solution 0.28 N. Later 1.5ml of Folin-Ciocalteu phenol solution 50% was added to the mixture. After incubation of the mixture in 35°C for 15 min, optical density (OD) was measured at 660 nm by spectrophotometer, which directly expresses the enzyme activity.

III. Results and Discussion

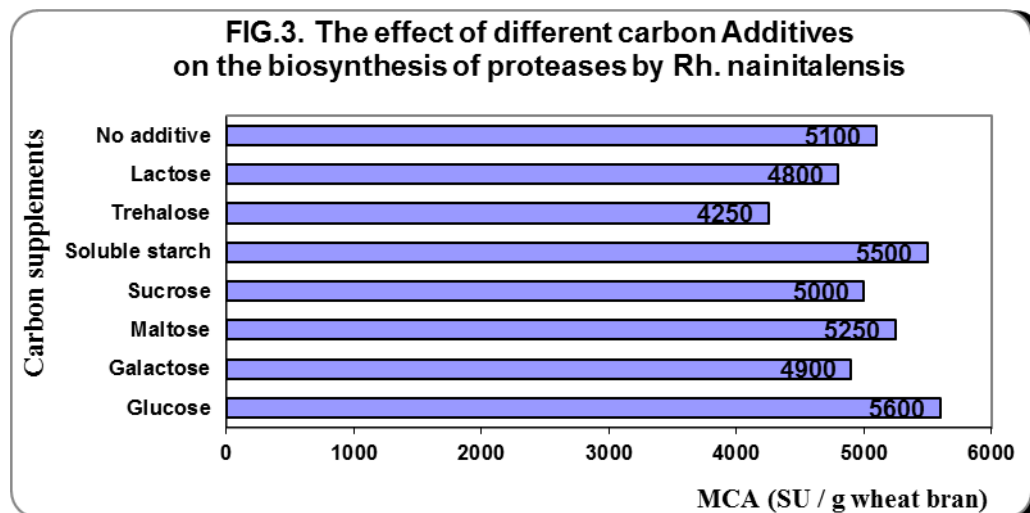
To measure the time taken to achieve maximum curdling potential, 28 metal trays containing the base culture medium (wheat bran at normal conditions) were prepared. After inoculation with spore suspension, four of them were selected for the measurement of curdling potential at the time zero (blank, the start of fermentation). The remaining 24 were placed in incubator at 35°C. Trays were taken out of incubator at 20 hour intervals, four at a time, and the enzyme activities were measured. The maximum enzyme activity was observed at 110th hour (Fig.1).



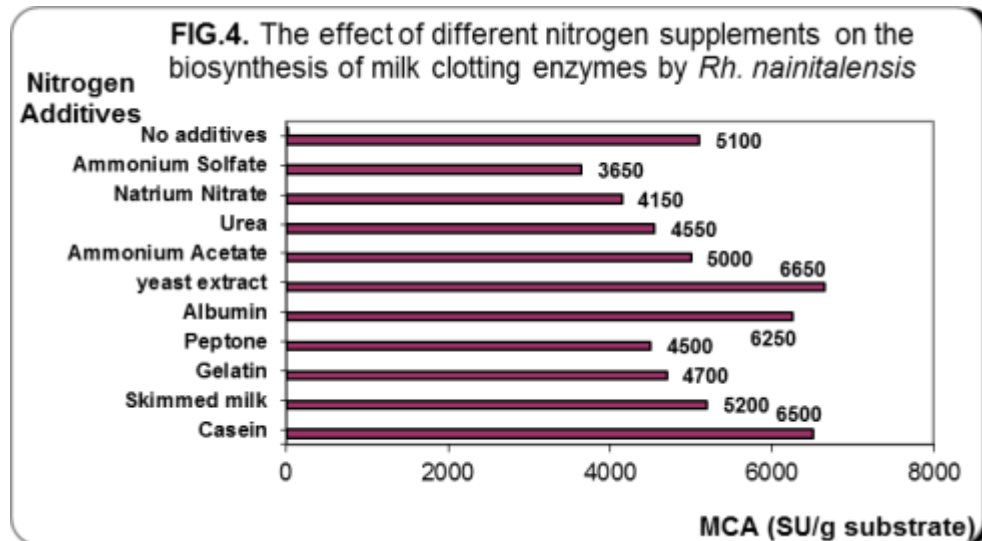
In addition, the substrate consisting only wheat bran was verified to be a better choice than those enriched by sorghum flour, rice flour, peanut flour or coconut powder and resulted in higher yields of enzyme compared to those basic culture media, which were enriched (10%) with other complex media.



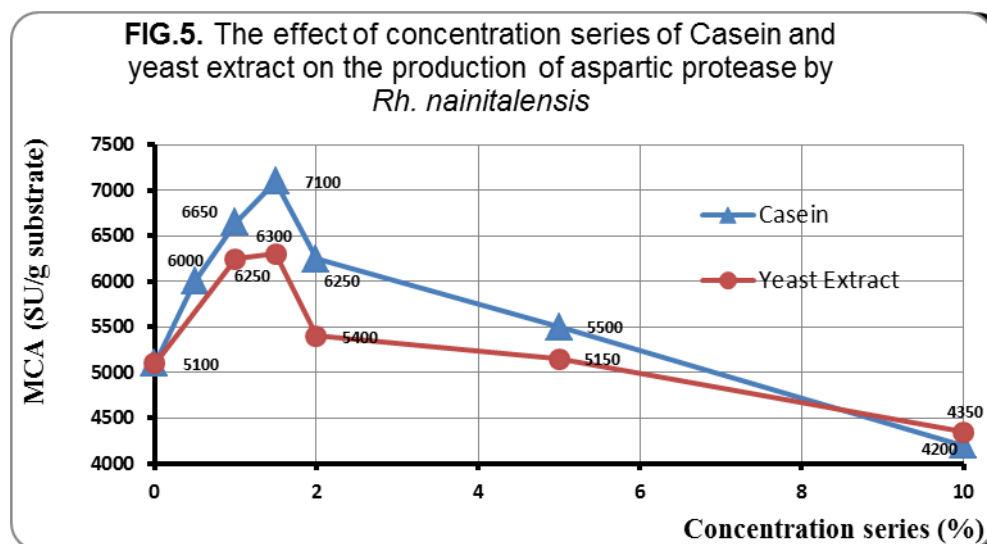
We then investigated the effect of adding additional sources of carbon to the fermentation system. We separately added 1% of lactose, fructose, sucrose, maltose, soluble starch, and trehalose to the system. Only starch caused a small increase in enzyme activity (Fig.3.).



An important factor in the growth of microorganism and production of enzyme is the availability of nitrogen to the microorganism. Nitrogen can be obtained by the microorganism either from organic or inorganic sources. Albumin, gelatin, casein, peptone yeast extract of nitrogen, urea, sodium nitrate, and ammonium were used as inorganic sources. 1% w/w of each organic source and 0.5% w/w of each inorganic source were separately added to the system and their effects on the enzyme yield were measured. When inorganic sources of nitrogen were added to the system, the enzyme yield was lower than the control, but higher when the organic sources of nitrogen were used. The highest increase caused by nitrogen source was achieved by casein (6650 SU/gr sub) followed by 6250 SU/gr sub for yeast extract (Fig.4.).



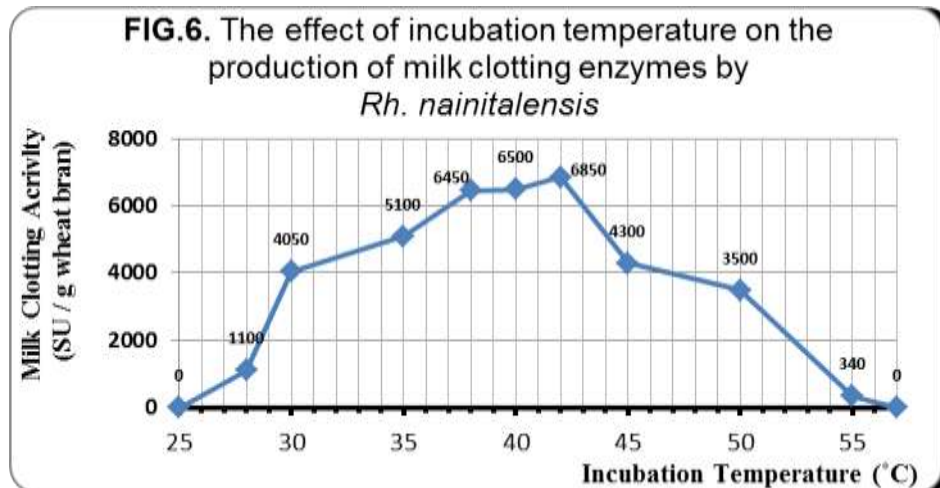
To find the pattern in which these sources of nitrogen affect the yield of enzyme, we added different concentrations of casein to the system (0.25 % to 10 %). Increasing the concentration of casein up to 1.5% resulted in higher yields; however the higher concentration resulted in a sharp yield decrease. At a concentration of 10%, the enzyme yield dropped to 4200 SU/gr sub. Addition of yeast extract up to 2% of the substrate's volume, improved the enzyme activity, 2-5% didn't increase the enzyme activity and further addition up to 10% decreased MCA to the point of 4200 SU/gr sub (Fig.5.).



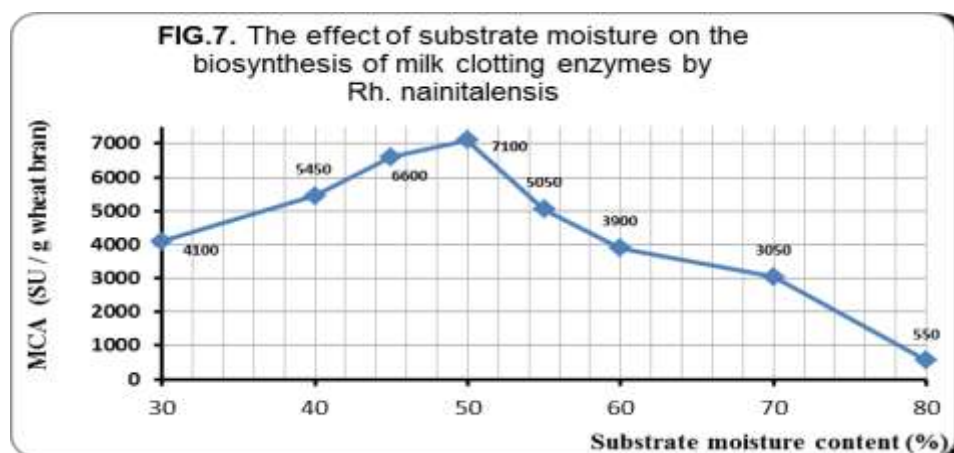
Furthermore, we studied the effect of macro elements on the enzyme activity. Sulfates of copper, iron, magnesium, and zinc were added to the system one by one and in combinations at different concentrations from 0.001 mg/L to 100 mg/L. No notable change of MCA was observed by adding Mg^{2+} , Fe^{2+} , Zn^{2+} , and Cu^{2+} . This may be an indication of adequate presence of macro elements in the wheat bran substrate.

The incubation temperature is one of most important factors affecting enzyme activity in all fermentation systems. Trays of SSF were placed in different incubation temperatures, between 25°C to 57°C, and enzyme yield in each temperature was measured.

Since *Rh. nainitalensis* is a thermophilic microorganism, we already expected a higher yield with the higher temperature of incubation. From 25°C to 42°C, growth of the microorganism increased considerably, with enzyme activity reaching 6500 SU/gr sub at 42°C, then it decreased sharply to the zero activity at 52°C.



In solid substrate (state) fermentation systems, humidity is another important factor affecting microbial growth and extracellular enzyme production. To measure its effect, MCA of enzyme yields were measured at the range humidity of 30% - 80%. We recorded the maximum yield (7000 SU/gr sub) at SSF humidity of 50%.



In the next step, we studied the effect of the initial culture inoculation on enzyme production. Spore Suspension at different concentrations, between 10^2 and 5×10^5 spores per gram of wheat bran, were prepared and used for the inoculation of substrate. The highest yield was recorded (6050 SU/gr sub) at a spore concentration of 10^3 per gram of wheat bran.

Considering all the factors tested optimum enzyme production conditions can be summarized as follows; Wheat bran substrate, 42°C, 50% humidity, 110-hour incubation time, inoculation of 10^3 spores per gram of wheat bran and 1.5% casein as nitrogen source additives. When these conditions were all fulfilled, a yield of 8400 SU/gr sub was achieved.

Because of impurities and additions in fermentation filtrate compare to the commercially available microbial rennet products, a direct comparison was not possible in this step. Therefore, we needed to perform a kinetic study of our rennet product before any conclusion. After a partial purification of the enzyme mixture, rennet characteristics such as specific enzyme activity, proteolytic activity, lipolytic potential, effect of Ca^{2+} concentration on the enzyme activity, optimal pH and optimal temperature with respect to the enzyme activity, resistance to temperature and pH changes, V_{max} and K_m , microbial infection and other characteristics would be investigated. In another part of our study the culture supernatant was semi-purified and the product revealed a specific curdling activity of 115000 SU/gr rennet powder [33].

A brief study of published results of milk curdling enzyme production shows superiority of SSF system over submerged fermentation system. Several researchers have confirmed superiority of curdling potential of the rennet produced by SSF to that produced by SF, with several fungal microorganisms being tested [34,35]. On the other hand there are studies which reported lower proteolytic potential for the rennet produced through SSF than the rennet produced through SF and from the same origin [36,37]. This could mean that SSF is a better choice for milk-coagulating enzymes used in cheese production industry. Our results contributed to the fact that the rennet produced from the fungal source *Rhizomucor nainitalensis* through SSF can be a commercially viable product considering its quality and the low costs involved.

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