Rennet production in cheese

TOPIC – Rennet Production

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INTRODUCTION

Rennet is a mixture of proteolytic enzymes (tissue and gastric enzymes) – rennin (chymosin) and pepsin, obtained from gastric mucous membrane of young ruminants [1]. These are the coagulating enzymes used in cheese production, but rennet is widely used. It is the oldest method of producing cheese. Rennin is present in its prorennin form with 42 amino acids at its N-terminal. Rennin (323 amino acids) is made of a single polypeptide chain with a molecular weight of 35, 000 Daltons. Rennin breaks the Phenylalanine-methionine bond in kappa casein [2]. The enzyme with high proteolytic activity is not beneficial due to the following reasons-

- It breaks down the milk protein casein imparting an unsatisfactory consistency to the cheese.
- It is undesirable as it leads to loss of digested protein with the whey.
- It results in bitter taste [3].

MICROBIAL RENNET

With the increase in demand for cheese with the rate of 4% a year and decrease in supply of calf rennet, its substitute plant and microbial rennet has captured the focus. *Mucor miehei, Cryptococcus albidus, Mucor pussilus, Bacillus cereus* and *Endothia parasitica* are some microbes which aid in the production of this conventional enzyme. Rennet from fungus *Mucor miehei* is involved in 33% of cheese production. It is preferred because of its high coagulation rate [4]. In the production process, the organism is grown in

nutrient medium, separated by filtration, followed by pH adjustment and then performing ultra filtration and vacuum evaporation [5].

PRODUCTION

Easily utilizable sugars such as glucose, lactose or sucrose can be used. Inorganic salts like Calcium, Phosphate and Magnesium are added to support the growth. Assimilable nitrogen source- Ammonium salts, Nitrate salts or amino acids are utilized. The nutrient concentration in the medium varies according to the species and strain [6]. The whey produced from the process is used in milk-based confectionery and for other processed foods [7].

The plant sources are papain from papaya tree and bromeline from pineapple [2]. Most plant rennet enzyme imparts bitter taste to cheese whereas the microbial enzymes are cheaper and easy to modify. The enzyme activity gets affected with the sugar source. More activity was shown with glucose and less with lactose, when *Mucor miehei* NRRL 3420 was used. *Mucor pusillus* QM436 clots milk with higher potential, indicating as a good substitute [4].

The animal and plant rennet have influence on the amino acid content of cheese. On the 60 [th] day of ripening, the Spanish and Portuguese cheese showed increase in total free amino acid (TFAA) content in cheese, representing > 50% of TFAA. The TFAA content was higher in cheese made with plant enzyme (854 mg/100 gm total solids) than with the use of animal enzyme (735 mg/100 gm of total solids). Cheese made with plant enzyme contained high levels of Met, Ser, Ile, Ala, His and Gln [8].

SUMMARY OF PATENTS-

The action of rennet is to cleave at a specified site in K casein and perform proteolytic function non-specifically. The rennet should have a low Proteolytic Activity (PA) and a high Milk-Coagulating Activity (MCA) i. e., a low PA/MCA ratio. If it is high then it makes the taste bitter, imparts a poor texture and reduces the yield of curd. But practically the microbial rennet has a high PA/MCA ratio than animal rennet. This patent focuses on increasing the MCA and decreasing the proteolytic activity by performing acylation on *Mucor pusillus* rennet. In this invention, the microbial rennet is succinylated with succinic anhydride which provides better safety and higher stability to the enzyme than maleic acid. The temperature should be between 0ËšC to 40ËšC and pH 4 to 10, preferably 7 to 9. After acylation the mixture is neutralized to 5 to 6. 5 and enzyme is obtained by gel filtration, ultra filtration or dialysis.

The preserved stomach of the calves is used to make a crude mixture of enzyme using water. The proenzyme is then activated to form the active version of enzyme. Filtration step is followed to clarify the enzyme and to obtain a concentrated form. Reclarification is done followed by filtration to remove the precipitated impurities. The enzyme is then added with salt and preservatives. The enzymes extracted from old and adult calves have low chymosin content.

Disadvantages of conventional rennet manufacturing process-

- High chymosin content requires young calves
- Labour intensive

- Time consuming

- Tedious clarifying process

- Large residual contaminated waste water

- Liquid rennet mixtures have low potential and results in transportation problem.

The enzymes are extracted using a weak DEAE-cellulose ion-exchanger, PEG exchanger and with the use of salts. The drawbacks of using them are-

- Difficulty in removing the PEG

- Costly

- Dissolution of salt is difficult, so the solution needs to be warmed.

Cultivation of yeast *Cryptococcus albidus, C. diffluens and C. aerius* provides the milk coagulating enzyme. The temperature should range from 15ËšC to 40ËšC with aeration and a pH of 3. 0–8. 0. The process works for 2-7 days. The enzyme is extracted by dialysis, salting out and freeze drying. Precipitation of mixture deprived of solids can be done with organic solvents, purification with ion-exchangers, salting out or low pressure. For salting out NaCl, $MgSO_4$ or NH_4NO_3 can be used. Further purification is done by performing dialysis against water. The non-dialyzable products are freeze dried and used. The enzymes obtained displayed high clotting activity and low proteolytic action.

The invention focuses on making low cost, high coagulating enzyme with low protease activity by making the use of yeast and their mutants. Yeast species of genus- *Torulopsis, Cryptococcus, Rhodotorula, Candida, Kluyveromyces,* their natural and artificially induced mutants are used. Strain of *Cryptococcus albidus* and its variant *aerius* were inoculated in

media containing 2. 0% glucose, 0. 1% yeast extract, 0. 1% Ammonium Sulphate, 0. 07% Sodium Phosphate dibasic, 0. 2% Potassium Phosphate monobasic and 0. 05% Magnesium Sulphate which was sterilized at 115ËšC for 20 minutes. It was incubated at 28ËšC for 72 hrs on shaker condition. Centrifugation was done and enzyme activity of the supernatant was determined which was Ca. 80 Soxhlet units/ml. Precipitation was done with Ammonium Sulphate followed by dialysis and liophilisation. The activity of powder was determined to be Ca. 140 rennet units (RU)/mg. This rennet was used to make parmesan cheese.

This patent aims on preservation and extraction of rennet from stomach of suckling calves. The rennet is recovered using a salt solution. The bovine calf stomachs are stored in a mixture of Ammonium Sulphate, Ammonium Chloride, Sodium Sulphate and Potassium Sulphate followed by refrigeration. The entire stomach is soaked in brine solution and refrigerated (4-5ËšC). Then they are kept in 3: 1 ratio of water and meat for weeks at 37ËšC.

The separation of rennet from *Endothia parasitica* and *M. miehei* was reported by Kobayashi et al. and its purification was carried using N-acetylpepstatin affinity gel columns. In this patent, Cibacron blue F3GGA, a soluble blue affinity ligand was used. It selectively binds to the enzyme at pH below 4. 0. It has no effect no microbial pigment. The dye on binding with the enzyme neutralizes three positive charges and therefore the isoelectric point reduces. In this case, the rennet precipitates in presence of excess dye which can be then centrifuged. It is again mixed with salt and chitosan, a polycation, used to eliminate the dye. Polyethyleneimine can also be used when the R group in the dye is polyethylene glycol. Two-phase process can

be used which involves Cibacron blue derived polyethylene glycol (CB-PEG) and dextran in water. This form two phases. The enzyme gets separated in the upper phase of CB-PEG. This portion is separated and added with NaCl to make two more phases.

In this patent, *M. miehei* NRRL 3420 was grown in nutrient medium. The filtrate was obtained and the pH was adjusted to 3 using conc. HCl. The filtrate was run on a precolumn containing polystyrene sulfonate to remove the pigments. An agarose bead support matrix was linked to Cibacron ligand was used. Sodium citrate of pH 5 eluted the rennet enzyme, giving 85% yield.

Along with the required enzyme i. e., rennet, other protein produces interference. For removing the non-specific enzymes, the filtrate is run over silicate column with a pH of 3 and 9. More than one absorbent can also be made in use. Rennet is stable at a pH range of 3 to 9. As the pH reduces, the stability decreases. The enzyme concentrate is obtained by precipitation or evaporation.

The clotting activity is determined by the following formula- 25X40/n X t u./mg

where, ' n '= quantity of rennet in mg/ml

' t '= clotting time

After fermentation, white powder is produced which is feasible to transport when compared to liquid form. But, the powder does not render optimum solubility. Addition of 2-3% of fatty acid monoesters of polyoxyethylene

sorbitan makes the rennet, obtained from *M. miehei,* stable. This ester contains 12-22 carbon atoms and 20 oxyethylene units/molecule. This mixture makes it suitable for storage, soluble in liquid, dust free and easy to handle.

The thermal destabilization property of rennet is beneficial as it can be used in pasteurized whey. Rennet shows low thermal destabilization when it is carbamylated with Potassium cyanate. If the whey has to be used for other purposes then the rennet activity should be restricted as it can lead to production of clumps and clots. The half-life of the enzyme is determined by-

$T = (t_2 - t_1) \ln2 / \ln A_1 - \ln A_2$

where, A_1 and A_2 are enzyme activity on heating at particular temperature at time t_1 and t_2. More the temperature, shorter is the half-life.

For obtaining microbial rennet, the sequence coding for rennet is expressed in expression vector through recombinant technique. It is then inserted into suitable bacterial host. Most favourable is *E. coli.* The host cells are grown under proper conditions. The cells are disrupted mechanically, enzymatically or through sonication. The suspension is allowed to centrifuge at 500-5000 g for 10 minutes-2 hours. The obtained pellet contains the desired enzyme. Denaturing agent is added to the pellet needing a pH of 7-8. Quanidine hydrochloride can be used. The mixture is allowed to undergo sulfitolysis. In this reaction, the disulfide bond is broken and sulfide is replaced with sullfonate. It's a nucleophilic reaction which results in protein-S-SO$_3$ (protein-S-sulfonate) formation. It is then subjected to weakly denaturing medium like

urea, which plays role in proper refolding. The extract is purified using molecular sieve or ion-exchange chromatography.

mRNA coding for rennet is extracted from the whole RNA content using oligodT column. Reverse transcriptase is used to make cDNA. Agarose Gel Electrophoresis is run to separate fragments of suitable size. With specific Restriction endonuclease cuts are made in fragments and vector. PolyG tail is added to the vector while polyC to cleaved fragment at termini. The cells are made competent with the help of Calcium Chloride and transformed cells will grow on tetracycline and ampicillin containing plate, if the cloning vector is pBR322. The plasmid from the transformed colonies is obtained by nucleases activity. Suitable host can be- *E. coli, B. subtilis* or yeast.

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