



Production of Cheese from Rennet enzyme using *Rhizomucor miehei* Isolated from cow dung

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Received: June 7th 2012, Accepted: June 15th 2012, Published: July 1st 2012

Abstract:

Rennet, a microbial coagulant, is obtained from *Mucor miehei* which is isolated from ruminant stomach. Cheese is one of the milk product which is produced from the enzyme rennet. The aim of the current work was to produce cheese from *Rhizomucor miehei* which is a common inhabitant of cow dung. Pure culture of the fungus was prepared. Further, the organism is grown in seed media as well as in production media for the production of rennet enzyme. The extract obtained from the filtration of production media was subjected to ammonium sulphate and acetone precipitations and centrifuged to obtain rennet in crude form. Ammonium sulphate precipitation was found to be more effective when compared to acetone precipitation in cheese production.

Key Words: Cheese, Rennet enzyme, *Rhizomucor miehei*.

Introduction:

Rennet is a complex of enzymes including a proteolytic enzyme (protease) found in mammalian stomach that coagulates the milk and is often used in the production of cheese. The active enzyme in rennet is called *chymosin* or *rennin*. Some molds such as *Rhizomucor miehei* are able to produce proteolytic enzymes. These molds are produced in a fermenter and then specially concentrated and purified¹. Cheese is a milk-based food product produced throughout the world in wide-ranging flavors, textures, and forms produced by coagulation of the milk protein casein. Typically, the milk is acidified and addition of the enzyme rennet causes coagulation. The solids are separated and pressed into final form².

Rhizomucor miehei is a species of fungus belongs to family mucoraceae. Homothallic forming numerous zygospores, which are reddish-brown to blackish-brown and globose³. It is commercially

used to produce enzymes which can be used for the production of cheese⁴.

Materials and methods:

Isolation of fungus: Fresh cow dung sample was collected and 1 gm of it was weighed and serially diluted and plated on PDA (Potato Dextrose Agar), streptomycin (0.1 mg/ml) was added to restrict bacterial growth and incubated at room temperature. Mixed and distinct fungal colonies were observed in 10⁻⁴ and 10⁻⁵ dilutions after 5 days.

Identification of fungus: Mixed colonies were stained individually using the lactophenol-cotton blue stain and observed under the microscope. The morphological structure observed under the microscope confirms the fungus to be *Rhizomucor miehei*. Pure culture of the fungus was then established on SDA (Sabouraud's Dextrose Agar) plates.

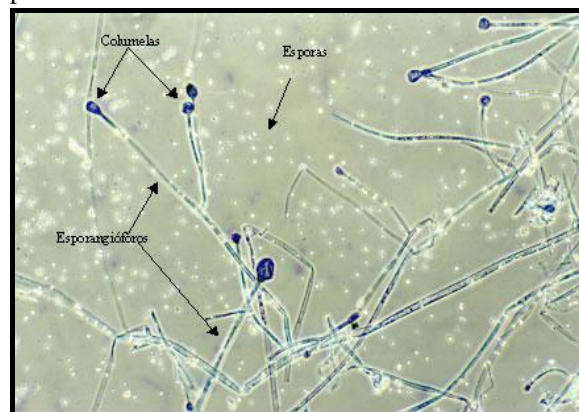


Fig.1. Microscopic view of *Rhizomucor miehei*

Establishment of seed media:

A colony was picked from the pure culture and used to inoculate in 250ml of seed media (glucose-20gm/ml, peptone-8gm/ml, NaCl-0.8gm/ml and pH 5.0-5.5)⁶. All the above constituents are mixed in a 500ml Erlenmeyer flask and this was incubated at 37°C. Mycelial mat was observed after 7 days which indicates the growth of the organism.

Establishment of production media:

Mycelial mat was removed and 1 ml of seed media was used to inoculate in 250ml of production media (glucose-20gm/ml, peptone-8gm/ml, casein-8gm/ml, KH_2PO_4 -2gm/ml, K_2HPO_4 -2gm/ml, FeSO_4 , FeCl_3 -0.02gm/ml, wheatbran-0.10gm/ml, corn steep liquor-0.01gm/ml, NaNO_3 -0.003gm/ml, CaCl_2 -0.1gm/ml)⁶. All the constituents are mixed in a 500ml of Erlenmeyer flask and this was incubated at 37°C. Mycelial mat was observed after 7 days.

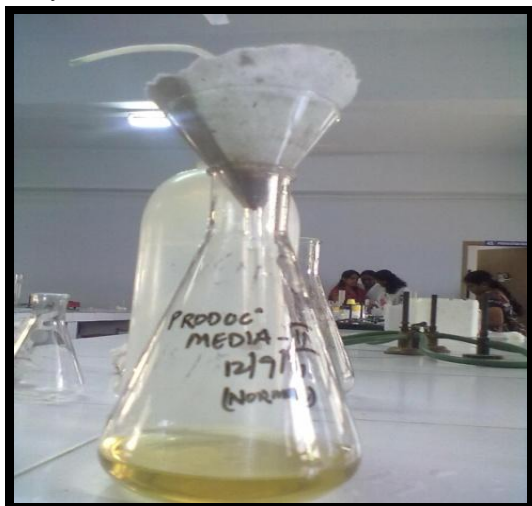


Fig.2. Production Media

Extraction of enzyme:

Mycelial mat was removed from the production media and the media containing the enzyme is filtered. The filtrate was divided into 2 equal halves of 100ml each. Ammonium sulphate and Acetone Precipitation was done for each of the halves respectively.

Ammonium sulphate precipitation:

Saturated ammonium sulphate was prepared by adding 80gms of ammonium sulphate to 100 ml of ice cold distilled water with constant stirring until it was completely saturated. Ice cold Saturated Ammonium Sulphate solution was added to the filtrate slowly with constant stirring. This was left undisturbed for few mins. Proteins were precipitated. The precipitate was further centrifuged at 10,000 rpm for 10 mins. Crude form of protein was obtained and dissolved in 1ml Tris buffer (1M) and preserved⁷.

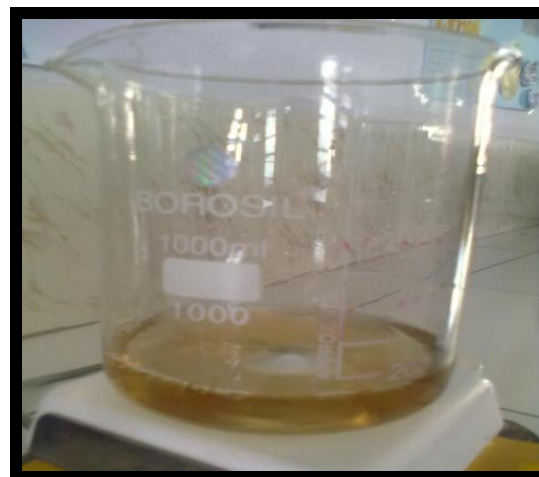


Fig.3. Ammonium Sulphate Precipitation of the Crude Extract

Acetone precipitation:

Ice cold acetone solution was added slowly to the filtrate with constant stirring under ice cold conditions. This was left undisturbed for few mins and further centrifuged at 10,000 rpm for 10mins. Crude form of protein was obtained and dissolved in 1 ml Tris buffer (1 M) and preserved⁸.



Fig.4. Acetone Precipitation of Rennet Enzyme

Production Of Cheese:

One liter of milk was boiled and cooled till lukewarm and it is divided into two halves. Rennet protein obtained after saturated Ammonium Sulphate Precipitation was added to 500ml of milk. The Rennet as obtained after Acetone Precipitation was added to another 500ml of milk. The two beakers were constantly stirred till curdling was observed after which it was allowed to stand for 2 mins. A clean muslin cloth was taken and the contents were filtered and collected separately.

Then it is tightly squeezed to remove the whey. Crude Cheese was retained on the muslin cloth. This cheese was collected into 2 petridishes and weighed separately.



Fig.5. Curdling of the Milk



Fig.6. Filtration of Milk



Fig.7. Cheese Ball

Results:

Fungi (*Rhizomucor miehei*) producing rennet enzyme was successfully isolated from cow dung sample and pure culture of it was established. Pure culture is used to inoculate seed and production

media. Mycelial mat was observed indicating the growth and the enzyme produced by fungus was released into the media. This enzyme was precipitated using protein precipitation techniques, rennet enzyme was isolated in Partially purified form. This form was used for curdling of milk to produce crude cheese. The cheese produced by the enzyme obtained by 2 different protein precipitation techniques was weighed. Higher quantity of cheese (280gms) was obtained by Ammonium Sulphate Precipitation than that with Acetone Precipitation (220gms). But, however the curdling of milk was found to be rapid with the enzyme obtained by acetone precipitation than that obtained by Saturated Ammonium Sulphate Precipitation.

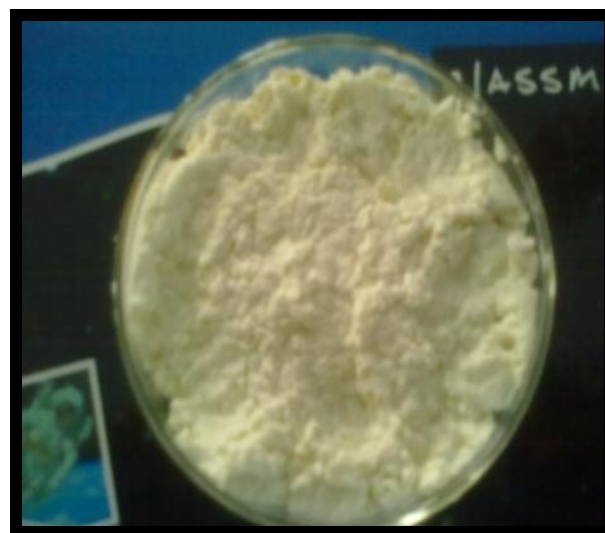


Fig.8. Crude Cheese

Discussion:

It is generally quite straight forward to locate the sources of enzymes; the natural sources of enzymes are where nature needs them⁹. Chymosin is also a very important industrial enzyme because it is widely used in cheese making. Chymosin is extracted from dried calf stomach for this purpose, but the cheese making industry has expanded beyond the supply of available calf stomach enzymes. Many proteases are able to coagulate milk by converting casein to paracasein and alternatives to chymosin are readily available. Rennet is the name given to any enzymatic preparation that clots milk¹⁰. Rennet is an extract from the fourth stomach of young ruminants, such as cows, goats, and sheep. This extract contains a

number of enzymes which are designed to help these animals digest their mother's milk, and when added to milk, rennet will cause the milk to coagulate, forming the curd and whey which are so essential in the cheese making process. It was discovered that the most active portion of the young animal's stomach to cause curdling was the abomasum, the last of the four chambers of the stomach of a ruminant animal. In particular, the abomasum from a suckling kid or calf was especially active. The abomasum was cut it into strips, salted and dried. A small piece would be added to milk in order to turn it into curds and whey. Sacrificing of the animal for this purpose is an ethical issue⁹. Scientists opted the usage of cow dung for the same purpose.

The milk components involved in cheese production is a soluble protein called casein. The enzyme rennet can be used to catalyze the conversion of casein in milk to para-casein by removing a glycopeptide from the soluble casein. Para-casein further coagulates, in the presence of calcium ions to form white, creamy lumps called the *curd*, leaving behind the supernatant called the *whey*⁹.

Casein --Rennet----> Para-casein (aq.) -- Ca⁺⁺--
--> Para-casein (Ppt-Cheese)

The precipitate is soft at this point and can be separated from the whey by the use of muslin cloth. Corn steep liquor and wheat bran which are the key components of the seed media and the production media, at industrial level mainly helps to enhance the growth of *Rhizomucor miehei* and rennet enzyme production which were not used in this experiment, however the yield of the enzyme and cheese were satisfactory comparable to the data available.

Conclusion:

In summary, the technology of processed cheesemaking, including analogues, has evolved dramatically over the past century. There is no doubt that increased consumption of processed cheese products worldwide is mainly due to consumer changes in food habits (i.e. popularity and acceptability of fast food and pizza). Our attempt was to produce good quantity of cheese in a short time by using Ammonium Sulphate Precipitation and Acetone Precipitation and the

cheese produced was found to be satisfactory in terms of quantity.

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