International Journal of Current Science Research
www.drbgrpublications.in

Volume: 2; Issue: 1; January-2016; pp 275-281. ISSN: 2454-5422

Submerged fermentation process for the production and characterization of milk clotting enzyme rennin from microorganisms

N. Valarmathi[1*], P. Periyakaruppiah[2] and M.Kannan[3]

[1]Department of Biotechnology, Kamaraj College of Engineering and Technology, Virudhunagar - 626 001, India

[2]Department of Botany, Yadava College (Autonomous), Madurai – 625 014, India

[3]Department of Microbiology, VHNSN College (Autonomous), Virudhunagar – 626 001, India

*Corresponding author E.mail: valarmathi170@gmail.com

Abstract

The Selected microorganisms were investigated for Milk Clotting Enzyme (MCE) production. The selected microorganisms (*Bacillus subtilis, Aspergillus niger and Aspergillus flavus*) were characterized by screening and biochemical test. The enzyme production and their milk clotting activity and their proteolytic activity of Rennin were investigated. Determine the protein contents of enzyme by SDS-PAGE and FTIR analysis. The results of milk clotting activity increased, *Aspergillus niger and Aspergillus flavus* than *Bacillus subtilis. Bacillus subtilis* was mutated by using ultra violet light. To compare the production of product by wild type *B. subtilis* and *B. subtilis* (uv mutated).

Keywords: Microbes, Rennin, Milk clotting activity, Proteolytic activity, SDS-PAGE, FTIR analysis, UV mutated (*B. subtilis*).

Introduction

Milk coagulation is the basic step in cheese manufacturing. Milk clotting enzymes are the primary active agents in cheese making; a very small amount of rennin is capable of coagulating a large amount of milk. The Soxhlet unit, which is still used in traditional cheese making, represents a conventional method to determine the total milk clotting activity of a rennin preparation. Clotting strength in Soxhlet units refers to the volume of raw milk which can be clotted by one volume unit of enzyme in 40 min at 35°C. At present microbial coagulants of fungal origin, which have been used in commercial cheese making since the 1960s, are of major importance. More than 100 fungal sources were reported by, (Foltmann 1959) which reflects the

high scientific interest in alternative coagulants for cheese production. Wheat bran was frequently used as main ingredient of the growth medium (Boopathy 1994).

Materials and Methods

Isolation and screening of Fungi

One gram of soil sample were serially diluted up to 10^{-7}dilution. The sample was plated on the potato dextrose agar (PDA) medium. The medium supplemented with 1% sterile skim milk at the time of plating. The plates were incubated for three days at room temperature. *Isolation and screening of Bacteria*

One ml of milk sample was serially diluted up to 10^{-7} dilution. The sample was plated on nutrient agar medium containing skim milk. Suitable dilutions of test material were plated and incubated at 37ºC for 48 hr.

Screening for milk-clotting enzymes

The mold isolated were transferred to the medium of (Gastrock *et al*., 1938) in which beer was replaced by 0.5 g of yeast extract per100 ml of medium, and were incubated for 3 days at 30ºC. The molds were cultivated at 30ºC for 5 days. The growth was then extracted with 50 ml of saline, and the milk-clotting activity was determined. In the bacterial screen, 100 ml of fresh milk (pH about 6.5) were added to 500-ml Erlenmeyer flasks, which were then sterilized by autoclaving at 15 psi for 20 min. The flasks were cooled to about 30ºC, and were inoculated with a saline suspension of bacterial isolates grown for 24 hr. The organisms were cultivated for 72 hr under submerged and surface conditions. In submerged culture, the inoculated flasks were kept at 30ºC on a rotary shaker (about 200rev/min). In surface culture, the flasks at 30ºC were not disturbed during the period of growth. Most of the organisms were pellicle-formers in surface cultures, and hence it was assumed that the majority of the cells were retained on the surface of the medium.

Growth Medium for submerged fermentation

To study effect of pH, carbon source, nitrogen source, experiments were carried out in Erlenmeyer flasks (250 ml) containing 30 ml of sterilized synthetic growth medium was inoculated with selected microorganisms. The cultivation media consisted of a carbon source, a nitrogen source, and a mineral solution which were sterilized separately. The production media consisted in 1% (w/v) of the carbon source and 0.5% (w/v) of the nitrogen source. Glucose, fructose, lactose and were tested as a carbon source while tryptone, casein and skim milk powder were tested as a nitrogen source. The fermentation medium contained corn steep solids (20g l^{-1}), NaCl (5gl^{-1}), $MgSO_4.7H_2O$ (5gl^{-1}), KH_2PO_4 (2gl^{-1}), $Caco_3$ (3gl^{-1}). Incubated rotary shaker for 7 days. The similar methodology used for bacteria and incubated rotary shaker for 4 days.

Bacterial Strain Improvement Method

Bacillus subtilis was mutated by using ultra violet light. To compare the production of product by wild type *B.subtilis* and *B.subtilis* (UV mutated).

Mutagensis by UV Radiation

4ml of cell suspension (10^8 cells/ml) of starting strain contained in a Petridis was placed under an ultra violet lamp (30W, Central electronic, USA) with a distance of 30cm and irradiated for different time interval between 20 and 180sec. The cell stirrer during irradiation. Treated cells were diluted in sterile physiological saline and 1ml of cell suspension was spread on to single colony isolation medium (its composition is same to slant medium), to calculate the lethality rate. The plates were incubated in dark at 37°Cfor 2days and mutants were isolated.

Assay for milk-clotting activity.

Milk-clotting activity was determined according to the method of Arima *et al* (1963) which is based on the visual evaluation of the appearance of the first clotting flakes, expressed in terms of Soxhlet units (SU).The clotting activity was calculated using the following formula

$$SU = \frac{2400 \times 5 \times D \times 0.5}{T}$$

Were *is, T* = clotting time (s)

D= dilution of test material

Assay for Proteolytic activity

Protease activity was determined by the according (Samal *et al.,* 1990). Absorbance was measured at 420 nm.

Purification of milk clotting enzyme

The enzyme having highest milk clotting activity was chosen for further purification studies using the modified method of (Otani *et al.,* 1959). The supernatant was treated with ammonium sulphate (40% saturation). The precipitated enzyme was collected by centrifugation at 3000rpm for 20 minutes in a refrigerated centrifuge was dissolved in minimum amount of distilled water.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)
SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done, using the method of Laemmli (1970).

Fourier transform infrared spectroscopy (FT-IR)

FTIR mainly used to determine the protein absorption range of enzymes. So the *Bacillus subtilis* (mutated) *and Bacillus subtilis* produced enzymes are subjected to the FTIR (Perkin Elmer spectrum RXI).

Result and Discussion

The fungal colonies (*Aspergillus flavus* and *Aspergillus niger*) were isolated from the soil sample. *Bacillus subtilis, Bacillus subtilis* (UV mutated), *Aspergillus niger* and *Aspergillus flavus* was cultivated under the submerged fermentation. Study effect the pH, carbon source, and nitrogen source. Experiments were carried out in Erlenmeyer flask (250ml) containing 30ml of sterilized growth medium and inoculated with isolated microorganisms. (Figure 1).

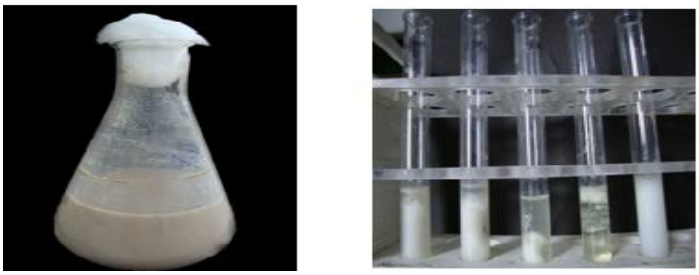

Figure 1.Sub merged fermentation and Milk clotting Activity

The milk clotting activity is irreversibly proportional to the proteolytic activity. The milk clotting activity increase and also decrease the proteolytic activity.In this study *B.subtilis* mutated milk clotting activity increase and decreases the proteolytic activity than the wild type *B.subtilis, A.niger* and *A.flavus* (Table 1).

Table 1.Milk clotting activity

Microorganisms	**Milk clotting activity (SU/ml)**	**Proteolytic activity 420nm**
Bacillus subtilis (UVmutated)	1.225±0.050*	0.11±0.03*
Bacillus subtilis (control)	1.110±0.040*	0.42±0.04*
Aspergillus niger	0.905±0.005*	0.39±0.06*
Aspergillus flavus	1.060±0.003*	0.32±0.02*

*± Std. error values of the samples (P=0.05) (n=5)

In this study, pH was found to be a prevailing factor affecting milk clotting enzyme production. The optimum initial pH value for maximum enzyme activity (pH 4.0). No milk clotting activity was dectable at (pH 3.0), under similar cultivation conditions. Determine the most favorable

source of carbon for enzyme production, either glucose or lactose were individually employed as a sole of carbon source in the production medium carbon sources stimulate the rennin production depending on the metabolism of the micro organisms under study, in the case of glucose for *B.subtilis* (mutated) fermentation. (Silveria *et al* 2005). Similarly present study maximum activity was observed when glucose used as a sole carbon source. The nitrogen source is influence the milk clotting enzyme by *B. subtilis* (mutated). Among the nitrogen source tested with casein and skim milk powder. Casein influence to increase the enzyme levels.

On other hand, the milk clotting activity was lowest when skim milk powder was the sole organic nitrogen source (Siliveria *et al* 2005), showed that casein played an important role in rennin production under the submerged fermentation. Poly acrylamide gel electrophoresis based on the findings of the enzyme produced by *B.subtilis* and *B.subtilis* (uv mutated) sample .*B.subtilis* and *B.subtilis* (mutated) showed the maximum milk clotting activity. Purified and their crude enzymatic extract, was precipitated obtained byfraction of 40 %.

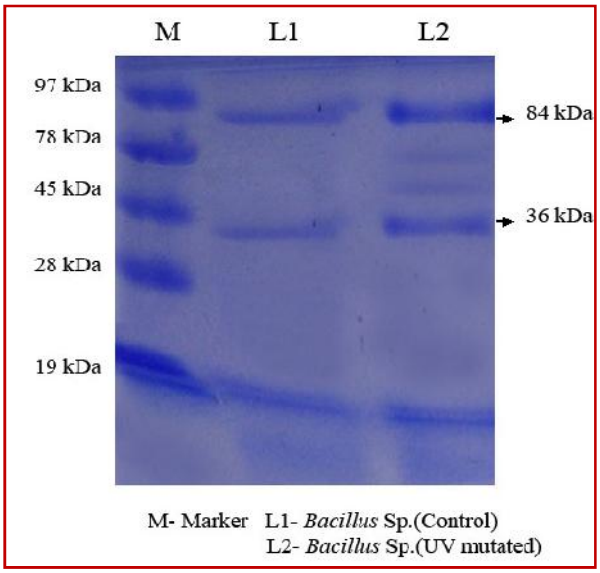

Figure 2. SDS–PAGE pattern of partially purified enzyme from microorganism.

FTIR spectroscopy works by shining infrared radiation on a sample and seeing which wavelengths of radiation in the infrared region of the spectrum are absorbed by the *Bacillus* control and *Bacillus* UV mutated. Characteristic bands found in the infrared spectra of proteins and polypeptides include the Amide I and Amide II The absorption associated with the Amide I band leads to stretching vibrations of the C=O bond of the amide, absorption associated with the Amide II band leads primarily to bending vibrations of the N—H bond (Figure 3). (Byler and Susi 1986, Surewicz and Mantsch 1988).

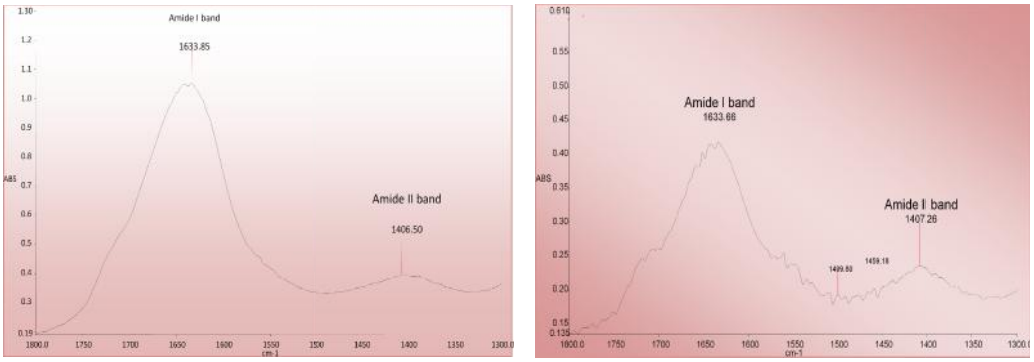

Figure 3. FTIR spectra of milk clotting enzyme from Bacillus Sp and Bacillus Sp (UV mutated)

Conclusion

The production of milk clotting enzyme by *A.niger, A.flavus, B.subtilis, and B. subtilis* (uv mutated) has been studied in submerged fermentation and optimized cultivation profile was reported. The mentioned microbial strain could be a valuable source of an enzyme having high milk clotting to tertiary proteolysis ratio and decreased resistance to thermal treatment. *Bacillus subtilis*, *Bacillus subtilis* (uv mutated). *Aspergilus niger, Aspergillus flavus* have ability to produce a milk clotting enzyme in submerged fermentation as opposed to solid state cultivation.

Reference

Arima K; Yu J and Iwasaki S 1970 Milk clotting enzyme from Mucorpusillus In: Perlman GE, Lorandl(eds) methods in enzymology, Academic press, New York.**19**: 446-459

Bhoopathy R *Indian Food Ind* 1994 13, 22–31. Dairy Sci. 76:2845-2855

Byler D; Michael and Heino Susi 1986 Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers* 25, no. 3: 469-487

Foltmann B 1959 On the enzymatic and the coagulation stages of the renneting process.*In Proceedings of the 15th International Dairy Congress, vol*. **2** (pp.655–661). London

Gastrock E A; N Porges; N Wells and A J Moyer 1938 Gluconic acid production on pilot plant scale. Ind. Eng. Chem. 30:782-789

Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. - Nature **227**: 680-685

Samal B B; Karan B and Stabinsky Y 1990 Stability of two novel serine proteinases in commercial laundry detergent formulations. Biotechnol. Bioeng. 35: 650-652

Silveira G G D; Oliveira G M D; Ribeiro E J; Monti R and Contiero J 2005 Microbial rennet produced by Mucor miehei in solid-state and submerged fermentation. *Brazilian Archives of Biology and Technology*, *48*(6), pp.931-937

Surewicz W K and Mantsch H H 1988 New insight into protein secondary structure from resolution-enhanced infrared spectra. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *952*, pp.115-130