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Study of proteolysis by *Micrococcus caseolyticus* isolated from refrigerated milk

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Abstract
Psychrotrophic bacterium produces thermo resistant enzymes that induce casein degradation and is the main cause of refrigerated raw milk spoilage. In the current investigation Micrococcus caseolyticus was isolated from refrigerated milk by serial dilution method and plate count was taken. psychrotrophic count was between 70-94 $\times 10^2$ CFU/ml. Among the bacterial isolates, *Micrococcus caseolyticus* was obtained and were further confirmed by using Bergey's manual of Determinative bacteriology (9th edition) and 16 S rRNA sequencing. *Micrococcus caseolyticus* showed highest proteolytic (0.237 mg/ml) at room temperature and it goes on decreasing with increase in temperature as well as at lower temperature. In the present study, the effect of EDTA was determined which proved to be the inhibitor of the enzymes produced by *Micrococcus caseolyticus*. The enzyme activity in the presence of EDTA was also maximum at room temperature (0.184) but significantly less compare to without EDTA. In conclusion, the prolonged refrigeration of milk at the dairy farm or at the dairy plant degrade the quality of milk due to proteolytic activity of psychrotrophic bacteria.

Keywords: psychrotrophic bacteria. proteolysis, enzyme inhibition

Introduction
Milk is an important part of human food and plays a prominent role in the Indian diet. The exact components of raw milk vary by species, but it contains significant amounts of saturated fat, protein and calcium as well as vitamin C. The casein content of milk represents about 80% of milk proteins. Psychrotrophic bacteria able to grow at 7°c or lower temperature, but had higher growth temperature requirement. Psychrotrophic bacteria found in diverse environment such as deep oceans, arctic regions and seasonally cold environment etc. These bacteria produce cold active enzymes with higher catalytic activities compare to mesic and thermic conditions. Now a days psychrotrophs are widely used in various biotechnological industries because they are able to produce heat stable hydrolytic extracellular enzymes such as proteases, lipases, phospholipases etc. In milk or dairy industries these enzymes degrade the quality of milk and causes spoilage of milk products. Psychrotrophs are dominant in prolonged milk storage. The psychrotrophic bacteria generally produce thermo-resistant enzymes which induce degradation of casein, and are the main cause of spoilage of refrigerated raw milk samples. Heat resistant proteolytic enzymes produced by psychrotrophs may be a serious problem in the production of sterile milk and other foods, and their destruction by heat is impractical. They significantly shorten shelf-life of heat-treated milk. Reduced yields and poor quality are prime concerns of the dairy industry because of the economic losses involved. so, the current study was carried to determine the proteolytic activity of psychrotrophic *Micrococcus caseolyticus* isolated from refrigerated milk. *Micrococcus caseolyticus* is a Gram positive non-spore forming cocci and can be distinguished from other Staphylococci phenotypically on the basis of their morphological characteristics. The genome size is much smaller (2.1 Mb) as compared to *Staphylococcus*.

Materials and Methods
Isolation of Psychrotrophic *Micrococcus caseolyticus*:
Psychrotrophic bacteria were isolated by serial dilution method. 1 mL of refrigerated raw milk was serially diluted in six tubes containing 9 mL of 0.1% sterile peptone water and 0.1 mL of each dilution was plated onto Plate Count Agar (M 1025) and incubated at 7°C for up to 10 days. After incubation isolated colonies were transferred on agar slants and their colony characters and biochemical characters were studied. *Micrococcus caseolyticus* was confirmed by using Bergey's manual of Determinative bacteriology (9th edition).

Molecular identification
Further identification was carried out by 16 s rRNA sequencing in which isolation of Genomic DNA was carried out using prepman ultra sample preparation reagent (Applied biosystem, Applera, USA). The Microseq 16s rRNA gene kit was used for PCR and sequencing. The facility was availed from molecular diagnosis, Zoology Department, Dr. BAMU, Aurangabad.

Generated sequences searched for the homologous sequences in NCBI database by using BLASTn. Gene bank accession numbers were obtained for the isolates. Phylogenetic tree of 10 closely related taxa was carried out by using MEGA X software.

Proteolytic activity

Psychrotrophic *Micrococcus caseolyticus* isolated form Plate Count Agar was inoculated on Milk Agar plates (M103) and incubated for 24 h at 37°C for the evaluation of proteolytic activity. The presence of clearing zones in Milk Agar plates indicating proteolysis was recorded. *Micrococcus caseolyticus* that produced halos in milk agar plates was inoculated in mineral medium (0.5 g/L NaCl, 0.4 g/L K_2HPO_4, 0.3 g/L KH_2PO_4) containing 10 g⁄L casein(pH 7.0) and incubated for up to 72 h at 30^0C in an orbital shaker at 120 rpm for enzyme production. A sample of the culture was then centrifuged at 1000 g for 15 min in 1.5 mL tubes to separate cell biomass and supernatant. The supernatant was used as crude enzyme for the analysis of proteolytic activity.

Table 1: Enzyme activity protocol

S No	Reagent	Blank (ml)	Standard (ml)
1	Casein	1	1
2	Phosphate buffer	1	1
3	Enzyme	0	1
4	Distilled water	1	0
Incubate at room temperature for 30 minutes			
5	TCA	3	3
Stand for 20 minutes and filter through Whatman Filter Paper no 42			
6	Filtrate	1	1
7	NaOH	1	1
8	Na2CO3	5	5
Stand for 10 minutes			
9	Folins reagent	0.5	0.5
Stand for 10 minute and Read the absorbance at 670 nm			

Effect of Temperature, protease inhibitor on proteolytic activity of *Micrococcus caseolyticus*

Crude enzymes were incubated in 0.1 M phosphate buffer pH 7.0 at 25^0C, 60^0C for 30 min, 75^0C, 100^0C for 30 s, and 120^0C for 10 s, and then proteolytic activity was measured as described above. The effect of protease inhibitors was determined after pre-incubation of the enzyme preparations with 5 mM EDTA for 10 min at 25^0C and then proteolytic activity was measured as described above.

Assay of Milk coagulation

Crude enzymes were filtered through Whatmann Filter Paper no 42 and mixed with UHT milk in sterile tubes to reach a final activity of 5 µ/ml. Tubes were incubated at 25^0C for up to 3 days. Milk coagulation was observed by visual inspection at different times. As a control, the enzymes were inactivated by heating at 100^0C for 15 min.

Results and Discussion

The quality of a food product is directly linked to the quality of the raw material used in its production. Storage of refrigerated milk at the dairy farm is a practice that aims to reduce milk spoilage due to the acidifying activity of mesophilic bacteria. The refrigerated storage of milk may enable the growth of psychrotrophic bacteria, which are destroyed through the conventional milk heating processes used at dairy plants. However, many psychrotrophic micro-organisms produce and secrete hydrolytic enzymes with spoiling effects. The spoilage of milk thus results in the production of many off-flavors which was characterized as fruity, musty, bitter, rancid and even putrid. Furthermore, their presence is not only linked to undesirable alteration in flavor, but residual activity of proteolytic enzymes can be related to gelation of milk and coagulation of milk proteins as well**.** In the current investigation, initially the psychrotrophic bacteria were isolated by serial dilution method and plate count was taken. The count of psychrotrophic bacteria showed value between 70-94 $\times 10^2$ CFU/mL for refrigerated milk samples. These organisms were further characterized by different morphological, cultural, and biochemical tests and confirmed by using Bergey's Manual of Determinative Bacteriology (9^{th} edition). The organisms obtained were *Enterobacter* species, *Pseudomonas* species, *Bacillus* species, *Stenotrophomonas* species, *Aeromonas* species *Micrococcus species*.

Identification of *Micrococcus caseolyticus*

In the present study *Micrococcus caseolyticus* was selected for proteolytic activity and identified on the basis of morphological, biochemical and molecular characters. The partial sequence of 16S rRNA gene of an isolate *Micrococcus caseolyticus* was carried out which contain 309 bases were given below.

GCACTCTGGCCGATCACCCACTCAGGTCGGCTATGTA
TCGTCGACTTGGTGAGGCGTTACCTCACCAACTAGCT
AATACACCGCGGGTGCATCTATAAGTGACAGCAGAA
CCGTCTATCACTATTGCTTCATGCGAAGCTAAATATT
AAGCGGTATTAGCTCCGGTATCCCGAAGTTATCCCA
GTCTTATAGGTAGGTTACCCACGTGTTACTCACCCGT
CCGCCGCTAACATCAGAGAGTGGAAGCTCTCTCGTC
CGTTCGCTCGACTTGCATGTATTAGGCTCGCCCACAG
CGTTCATCCTGAGCG

The phylogenetic position of 10 taxa including isolate in relation to *Micrococcus* genus in Gene Bank is given below

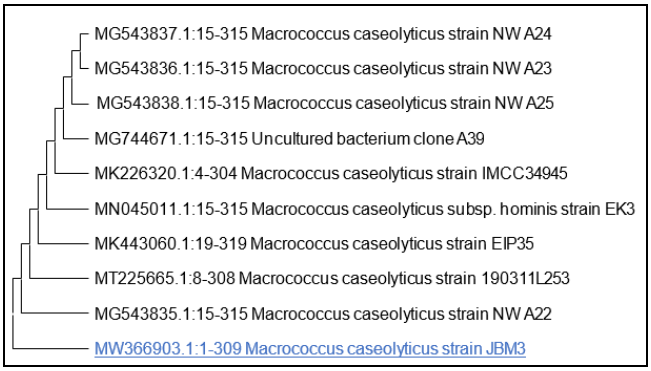

Fig 1: Phylogenetic position of isolated *Micrococcus caseolyticus* accession no MW366903 with closely related 10 taxa.

Proteolysis was determined at different temperature values. EDTA was used as protease inhibitor and then proteolytic activity was studied as per the previous protocol. All the results were compared with original proteolytic activity of organisms among these at room temperature highest proteolytic activity was observed (0.237 mg/ml) in the absence of enzyme inhibitor EDTA. Proteolytic activity was also determined in the presence of enzyme inhibitor EDTA, which also showed highest activity at room temperature (0.184 mg/ml) but enzyme activity significantly decreased. Results are summarized in table 2 and graph 1.

Table 2: Enzyme activity of Micrococcus caseolyticus at different temperatures with and without EDTA (enzyme inhibitor).

S No	Temperature	Enzyme activity without EDTA	Enzyme activity without EDTA
1	10^0C	0.154	0.132
2	25^0C	0.237	0.184
3	50^0C	0.126	0.095
4	75^0C	0.085	0.056
5	100^0C	0.046	0.023

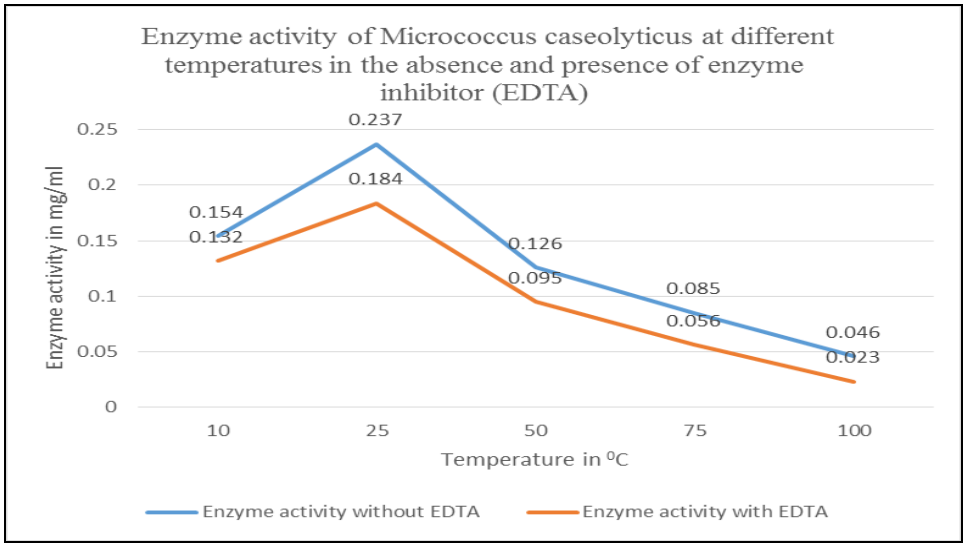

Fig 2: Enzyme activity of Micrococcus caseolyticus at different temperatures in the absence and presence of enzyme inhibitor (EDTA).

The inhibition by EDTA indicates that the enzymes belong to the metallo protease group of proteinases. Micrococcus caseolyticus showed, very less inhibition at 100 ^{0}C (0.023). Lastly milk coagulation was carried out which showed that, the addition of crude proteases to sterile milk (commercial UHT milk) resulted in extensive coagulation within 3 days at room temperature. This result indicates that psychrotrophic bacteria can effectively cause spoilage in milk and dairy products. All the results of current study coincide with the results obtained by Maria F B L Nornberg, Rosanes S C Friedrich, Rita D N Weiss, Eduardo C Tondo.

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