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Cold-active hydrolytic enzymes production by psychrotrophic *Staphylococcus eqourum* strain QCM1 isolated from refrigerated milk

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Abstract
Psychrophiles are the most abundant extremophiles in terms of biomass, diversity, and distribution. Cold-adapted microorganisms can be found in large areas of the planet, including the oceans 70 percent and the Polar Regions (20 percent). Man-made habitats (such as refrigeration and freezer systems) make up a small portion of the total number of potential habitats for cold-adapted organisms. In recent years, there has been a lot of research into the diversity of microorganisms that live in cold environments, with a focus on culture-dependent and culture-independent techniques. In the current investigation *Staphylococcus eqourum* was isolated from refrigerated milk by serial dilution method and plate count was taken. psychrotrophic count was between 60-91 $\times10^2$ CFU/ml. Among the bacterial isolates, *Staphylococcus eqourum* was obtained and were further confirmed by using Bergey's manual of Determinative bacteriology (9th edition) and 16 S rRNA sequencing. *Staphylococcus eqourum* showed significant hydrolytic potential with different temperatures and pH. Optimum proteolytic activity of 43 U/ml at 20^0C pH (7) was showed by bacterium. It goes on decreasing with increase in temperature and pH as well as at lower temperature and pH. Lipolytic activity of *Staphylococcus eqourum* strain QCM1was observed as 18.3 U/ml at 20^0C and 18.6 U/ml at pH (7). Our results suggest that *Staphylococcus eqourum* strain QCM1 is capable of producing hydrolytic cold active enzymes and can be studied further for investigation of their hydrolytic enzymes.

Keywords: psychrotrophic bacteria. proteolysis, lipolysis, enzyme assay

Introduction

Milk is an essential component of human nutrition, and it plays a significant role in the Indian diet. Raw milk contains significant amounts of saturated fat, protein, and calcium, as well as vitamin C, depending on the species. In terms of biomass, diversity, and distribution, psychrophiles are the most abundant extremophiles. Cold-adapted micro organisms can be found in large areas of the planet, including the oceans 70 percent and the Polar Regions (20 percent). Man-made habitats (such as refrigeration and freezer systems) make up a small portion of the total number of potential habitats for cold-adapted organisms (Margesin *et al* 2010). Many studies on bacterial diversity using snow, ice, water, soil, and sediment samples of the alpine cold habitats have revealed that psychrophiles are predominant in these habitats (Xang etal 2005, Lin 2007, Ettoumi 2009, Prasad 2014) [4, 17]. In recent years, there has been a lot of research into the diversity of microorganisms that live in cold environments, with a focus on culture-dependent and culture-independent techniques. 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 (Gupta *et al* 2004). Microbes that survive in low-temperature environments are important for their metabolic contributions to the ecosphere, as well as their enzymes that could be used in industry (Gounot 1991; Margesin and Schinner 1994) [6]. Psychrotrophs are the most common bacteria found in milk that has been stored for a long time. The main cause of spoilage of refrigerated raw milk samples is psychrotrophic bacteria, which produce thermo-resistant enzymes that cause casein degradation. Heat-resistant proteolytic enzymes produced by psychrotrophs could be a serious problem in the production of sterile milk and other foods, and destroying them with heat is not practical. They significantly reduce shelf-life of heat-treated milk. The current study was carried to determine the hydrolytic potential of psychrotrophic bacterial isolate QCM1 isolated from refrigerated milk.

Materials and Methods

Isolation of Psychrotrophic *Staphylococcus eqourum*

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. *Staphylococcus eqourum* 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

After 48 hours of incubation, a clear zone of skim milk hydrolysis indicated the presence of protease producing colonies. On the basis of clear zone size, five colonies were chosen for protease production. The production of proteases was carried out in a medium that contained 10 g L^{-1} glucose, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 1 g L^{-1} KH_2PO_4, and 0.2 g L^{-1} $MgSO_4$. $7H_2O$. The pH of the autoclaved broth was adjusted to 7.0 by adding sterilized Na2CO3 solution (20% m/v). The media was inoculated at 1.0% v/v with 24 h old culture and incubated at 15 ± 2 ^{0}C in a refrigerated incubator shaker (Scigenics Bio- tech., India) at 100 rpm/ min for 48 h. The growth cultures were then centrifuged at 4^{0}C at 10000 rpm for 15 minutes and the supernatant was used for protease assay. One isolate QCM1 was chosen for further investigation because it produced the maximum enzyme.

Optimization of protease production

The experiments were performed in twice, with the outcomes of the two separate observations averaged. The broth media were inoculated with the organism and incubated at 20^{0}C to decide the optimum time period for cold-active protease production at pH (7). The enzyme activity was assessed after the samples were taken at varying intervals. The inoculated broth was incubated at varying temperatures to determine the optimal temperature at (pH 7). The best pH condition for fermentation was determined by inoculating the broth with a specific pH. The inoculated media was incubated in a static condition and in a rotary shaker at 120 rpm/min to determine the best pH condition for fermentation. The effect of inducers was explored by applying multiple inducers (bovine serum albumin, casein, skim milk, or egg albumin) to the media at a rate of 0.5 % to 1 %.

Lipolytic activity

The isolate QCM1 was cultivated in the medium consisting of peptone (3%), NaCl (0.5%) and tributyrin (1%) (w/v) pH 7.0, 15 °C, 120 rpm for 24 hours [h]. The extracellular culture broth was used as enzyme source. The media was optimized by using olive oil (1% w/v), NaCl (0.5% w/v) and peptone (3% w/v). Lipase activity was measured by spectrophotometric method using paranitro phenyl palmitate (pNPP) as substrate. For optimization of temperature for enzyme catalysis, crude enzyme was assayed over different temperatures (5-55°C) at pH 7.0 in 50 mM phosphate buffer. To determine optimum pH, enzyme was incubated at optimized temperature in the pH range of 2.0-10.0. The following buffers were used: pH 2.0-5.0 citrate phosphate buffer, pH 6.0-8.0 phosphate buffer and pH 9.0-10.0 carbonate bicarbonate buffer. For enzyme assay, 0.1 ml of enzyme was added to a 0.9 ml of pNPP substrate solution (2 mg /ml in isopropanol) and 1 ml phosphate buffer (50 mM, pH 7.0). The mixture was incubated for 1 hour at optimized conditions. The amount of para- nitrophenol (pNP) liberated was measured spectrophotometrically at 410 nm (Winkler and Stuckmann, 1979). One unit (U) of lipase activity is defined as the amount of enzyme necessary to hydrolyze 1 µmol of pNPP /min under assay conditions.

Effect of pH and Temperature on lipase stability

Thermal stability of crude enzyme was investigated by incubating the enzyme in phosphate buffer (50 mM, pH 7.0) at different temperatures ranging from 5-50°C for 1 h and aliquots were assayed for residual activity. The pH stability was tested by incubating the enzyme at 10°C in the pH range of 5.0-10.0 using different buffers at 50 mM concentration for 1 h. Buffer systems used were the same as mentioned earlier. The aliquots were assayed for residual enzyme activity. All experiments were done in triplicates.

Results and Discussion

Isolation and identification of proteolytic psychrotrophic bacteria

The activity was compared to the existence of psychrotrophic proteolytic bacteria in refrigerated raw milk, which was revealed by microbiological experiments. After 7 days of incubation at 20^{0}C, 5 distinct bacterial colonies expressing extracellular protease on milk agar media were isolated from refrigerated milk obtained from Quality Milk dairy in Aurangabad. Three possible isolates, QCM1, QCM3, QCM5 were chosen for hydrolytic potential at varying temperature and pH based on the skim milk hydrolysis zone around the colonies. One of the isolates, QCM1, was identified on the basis of morphological, biochemical characters and confirmed by using Bergey's manual of Determinative bacteriology (9th edition) as well as 16S rRNA sequencing as *Staphylococcus eqourum* strain QCM1 and received accession number MW412938 from NCBI Gene Bank. The partial sequence of 16S rRNA gene of an isolate *Staphylococcus eqourum* strain QCM1 was carried out which contain 275 bases were given below.

TTGCTCCTTTGAAGTTAGCGGCGGACGGGTGAGTA
ACACGTGGGTAACCTACCTATAAGACTGGAATAAC
TTCGGGAAACCGGAGCTAATGCCGGATAACATTTG
GAACCGCAGGTTCTAAAGTAAAAGATGGTTTTGCT
ATCACTTATAGATGGACCCGCGCCGTATTAGCTAG
TTGGTAAGGTAACGGCTTACCAAGGCAACGATACG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGAA
CTGAGACACGGTCC AGACTCCTACGGGAG

The phylogenetic position of 10 taxa including isolate in relation to *Staphylococcus eqourum* strain QCM1 in NCBI Gene Bank is given below.

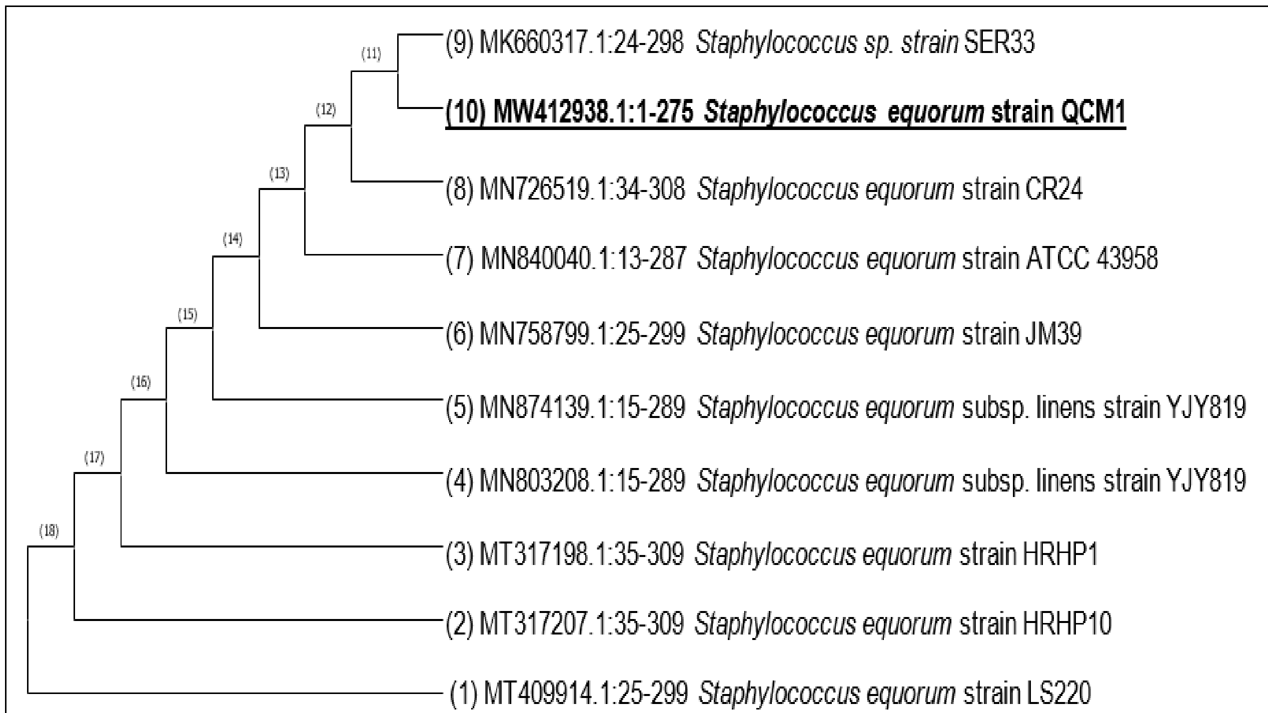

Fig 1: Phylogenetic position of isolated *Staphylococcus eqourum* strain QCM1 accession no MW412938 with closely related 10 taxa.

Effect of temperature and pH on Protease production

Temperature has a significant impact on bacteria's' ability to produce protease. The cells were incubated at 5–50 ^{0}C in protease production medium to evaluate the influence of temperature on enzyme production while keeping all other parameters stable. Maximum production, 43.0 U mL⁻1, was found at 20^{0}C (Fig. 2A). Increases in incubation temperature above 20^{0}C resulted in a steady decrease in enzyme activity, which was fully inhibited at 45^{0}C. The findings showed that a large amount of enzyme produced at temperatures between 15^{0}C and 25^{0}C. As a consequence, it's advisable that for industrial purposes, minimal temperature gradient in enzyme production is preferable. The pH of a culture has a major impact on enzymatic processes and compound transfer through the cell membrane. The maximum enzyme production was obtained at pH (7), 43.0 U mL–1, after 5 days of incubation (Fig. 2B).

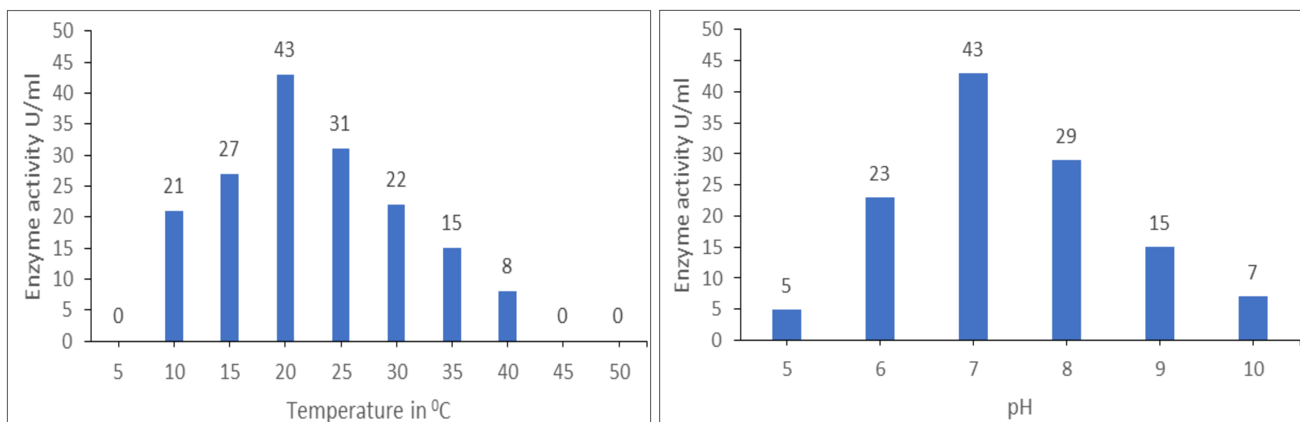

Fig 2: (A) Effect of temperature on protease production. (B) Effect of pH on protease production.

Effect of temperature and pH on lipase production

Optimized medium containing olive oil (1% w/v), NaCl (0.5% w/v) and peptone (3% w/v) was used to determine optimum enzyme activity at different temperature and pH. The effect of temperature was measured by using the standard assay protocol at temperatures varying from 10-55 ^{0}C and pH (5-10). The enzyme was pre-incubated at different temperatures for 3 hours to evaluate the protease stability with temperature changes, and enzyme activity was calculated as per standard protocol. Maximum lipase activity of 18.3 U/ml was observed when the isolate *Staphylococcus eqourum* QCM1 was grown at 20°C (Figure 3A). Earlier, optimum temperature of 28°C was reported for *Halomonas* sp. (Dang *et al.*, 2009). Citrate phosphate (pH5–6), sodium phosphate (pH7), Tris–HCl (pH 8) and glycine–NaOH (pH9–11) were used to determine the optimal pH for enzyme activity. The activity of reaction mixtures was measured after they were incubated for 30 minutes at 20^{0}C. Pre-incubating protease without substrate at different pH values (5–10) for 1 hour at 20^{0}C was used to determine its stability. At each exposure, the residual enzyme activity was calculated using a standard assay. Optimum pH for lipase production was found to be 18.6 U/ml at pH 7.0 (Figure 3B).

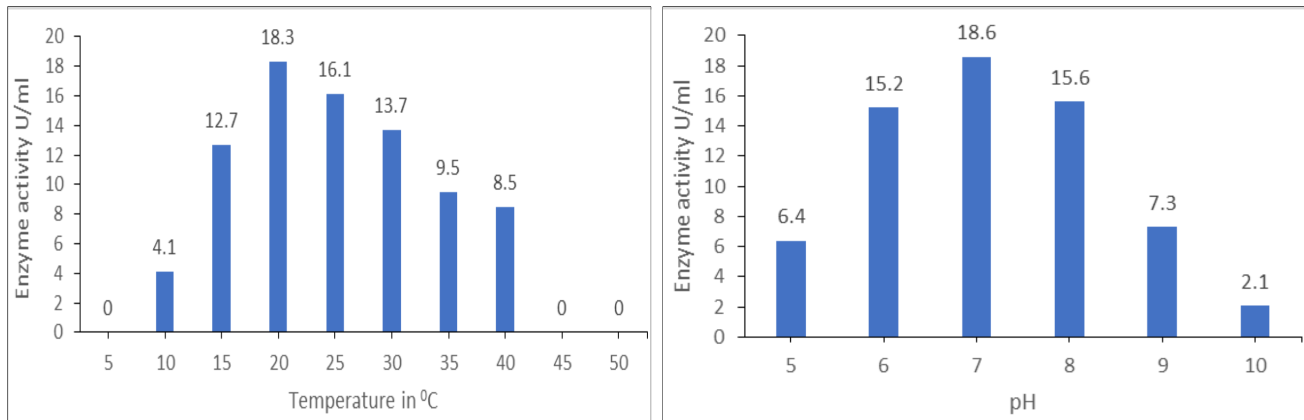

Fig 3: (A) Effect of temperature on lipase production. (B) Effect of pH on lipase production.

According to the current microbial research, refrigerated milk may be a rich source of technologically important microbes. Like many hydrolytic enzymes, the protease and lipase from *Staphylococcus eqourum* QCM1 is secreted largely at the late exponential phase of growth with maximum production at 20^0C and pH 7.0 (Baghel *et al.* 2005). The protease and lipase from *S. eqourum* QCM1 showed maximum activity at 20^0C, and thus can be classified as a cold-active hydrolytic enzyme (Morita 1975). As a consequence, implementing *S. eqourum* hydrolytic enzymes in detergent industries may increase the removal of protein and fats containing stains significantly. Psychrophilic enzymes have greater activity at low to moderate temperatures than their mesophilic counterparts, and their biotechnological potential has prompted increased interest in recent years.

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Conflict of Interest
The Authors declare no conflicts of interest.

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