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ISOLATION AND CHARACTERIZATION OF *ORNITHINIMICROBIUM HUMIPHILUM SSOC1* FROM REFRIGERATED MILK AND EVALUATION OF ITS PROTEOLYTIC ACTIVITY

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ABSTRACT

Cold-adapted microorganisms are widely distributed in nature and form a major part of the Earth's microbial population. Artificial cold environments such as refrigeration systems represent a small part of their total habitats. The present study aimed to isolate psychrotrophic bacteria from refrigerated milk and evaluate their ability to produce hydrolytic enzymes. The psychrotrophic count ranged from 58–85 × 10^2 CFU/ml using the serial dilution and plate count method. Among the isolates obtained, was identified based on morphological and biochemical characteristics with reference to Bergey's Manual of Determinative Bacteriology (9th edition). The identification was further confirmed by 16S rRNA gene sequencing. The isolate showed enzyme production at different temperatures and pH levels. Maximum protease activity (0.28 U/ml) was observed at 20°C and pH 7. The activity decreased at temperatures and pH values above or below this optimum. These results indicate that *Ornithinimicrobium humiphilum SSoC1* can produce cold-active protease enzymes and may have potential industrial applications.

KEYWORDS: Psychrotrophic bacteria, 16 S rRNA sequencing, proteolytic activity.

INTRODUCTION

Milk is a highly nutritious biological fluid and an essential component of the Indian diet, providing proteins, lipids, minerals, and vitamins. Owing to its rich nutrient composition, raw milk serves as an excellent growth medium for microorganisms. Although refrigeration is widely employed to extend the shelf life of milk, psychrotrophic bacteria are capable of proliferating at low temperatures and represent a major cause of spoilage during cold storage.

Psychrophiles and psychrotrophs constitute one of the most abundant groups of extremophilic microorganisms in terms of biomass, diversity, and global distribution. Approximately 70% of the Earth's surface is covered by oceans and nearly 20% by polar regions, both of which represent permanently or seasonally cold environments. In contrast, artificial cold habitats such as refrigeration systems constitute only a minor fraction of cold ecosystems (Margesin et al., 2010). Several investigations on microbial diversity from snow, ice, glacier sediments, and alpine soils have demonstrated the predominance of cold-adapted bacteria in such environments (Xiang et al., 2005; Lin et al., 2007; Ettoumi et al., 2009; Prasad et al., 2014).

Cold-adapted microorganisms have attracted considerable attention due to their ability to synthesize extracellular hydrolytic enzymes that remain active at low and moderate temperatures. Enzymes such as proteases, lipases, and phospholipases produced by psychrotrophs possess high catalytic efficiency at low temperatures, making them valuable for industrial applications including food processing, detergents, pharmaceuticals, and environmental biotechnology. These enzymes are energy-efficient and environmentally sustainable alternatives to mesophilic counterparts.

Despite their biotechnological importance, psychrotrophic bacteria pose a significant challenge in the dairy industry. During refrigerated storage, these organisms produce heat-stable proteolytic enzymes that survive pasteurization and ultra-high temperature processing. Such enzymes degrade casein and other milk proteins, leading to bitterness, gelation, off-flavors, and reduced shelf life of processed milk products (Gupta et al., 2004). The thermostable nature of these proteases makes their complete inactivation difficult using conventional heat treatments, thereby affecting product quality and economic value.

Therefore, the present study was undertaken to isolate psychrotrophic bacteria from refrigerated raw milk and to characterize the proteolytic activity of isolate SSoC1. The study aims to evaluate its enzymatic potential under varying temperature and pH conditions and assess its relevance in dairy spoilage as well as possible industrial applications.

MATERIALS AND METHODS

Isolation of Psychrotrophic *Ornithinimicrobium humiphilum* 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. *Ornithinimicrobium humiphilum* 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 SSoC1 was chosen for further investigation because it produced the maximum enzyme. All experiments were conducted in duplicate and mean values were calculated. The optimum incubation period for protease production was determined by incubating the inoculated production medium at 20°C and pH 7, followed by estimation of enzyme activity at regular time intervals.

The effect of temperature (5–50°C) on protease production was evaluated while maintaining pH 7. The influence of pH (4–9) was assessed by adjusting the initial pH of the medium and incubating at the optimized temperature. To determine the effect of aeration, cultures were incubated under static conditions and in a rotary shaker at 120 rpm. The role of proteinaceous inducers, including bovine serum albumin, casein, skim milk, and egg albumin (0.5–1%, w/v), was investigated to assess their influence on enzyme production.

RESULT AND DISCUSSION

The isolate SSoC1 produced small (≈1 mm), circular, convex, white and opaque colonies with entire margins and a mucoid texture. Microscopic examination revealed Gram-positive, non-motile, short rod-shaped, non-spore-forming cells without PHB granules. These characteristics are consistent with previously reported descriptions of *Ornithinimicrobium humiphilum* (Groth et al., 2001). Biochemical analysis showed acid production from glucose, arabinose, mannitol, fructose and sucrose without gas formation. The isolate was catalase positive but negative for oxidase, urease, gelatin hydrolysis, indole production, methyl red reaction and nitrate reduction. Similar biochemical profiles have been documented for *O. humiphilum* and related species (Mayilraj et al., 2006; Kämpfer et al., 2011).

Molecular identification based on 16S rRNA gene sequencing confirmed the isolate as *Ornithinimicrobium humiphilum*. The partial sequence (398 bp) was submitted to GenBank under accession number MW412939. The findings are in agreement with earlier taxonomic descriptions and confirm the identity of strain SSoC1 (Stackebrandt and Goebel, 1994).

16S rRNA partial gene sequence of isolate SSoC1 identified as *Ornithinimicrobium humiphilum* (Accession Number:MW412939).

CTTCAGCGACGCCGCGTGCGGGATGATTGCCTTCGGGTTGAAAACAGCTTTCAGCTCTGACGAATCTTTTGTGAC
CGTAGGAGCAGAAGAAGCACGGGCTAACCACGTGCCAGCAGTGGCGGTAACACGTAGGGTGCCAGCGATGTCC
GGAATAATTGGGCGTAAAGAGCTAGTAGGTGGCTTGTCGCGTCTGCTGTGAAAACCCGGGGCTTAACCCCGGAC
TTGCAGTGGGTACGGGCAGGCTAGACAGTGGTAAGCGAGACTGGAGATCCTGGTGTACCTGTGGAATGCCTAGA
TATCATGATGAAGACCGATGGCGAAGGCAGGTCTCCGGGCCATTACTTACGCTGAGAAGCGATAGCGTGGGGAG
GCAACAGGATTTGATACCCTTGTGGTTC

Phylogenetic position and relationship of 10 taxa along with isolate SSoC1 (MW412939) in relation to species of the genus *Ornithinimicrobium* found in the NCBI GeneBank database was illustrated in Figure 1.0

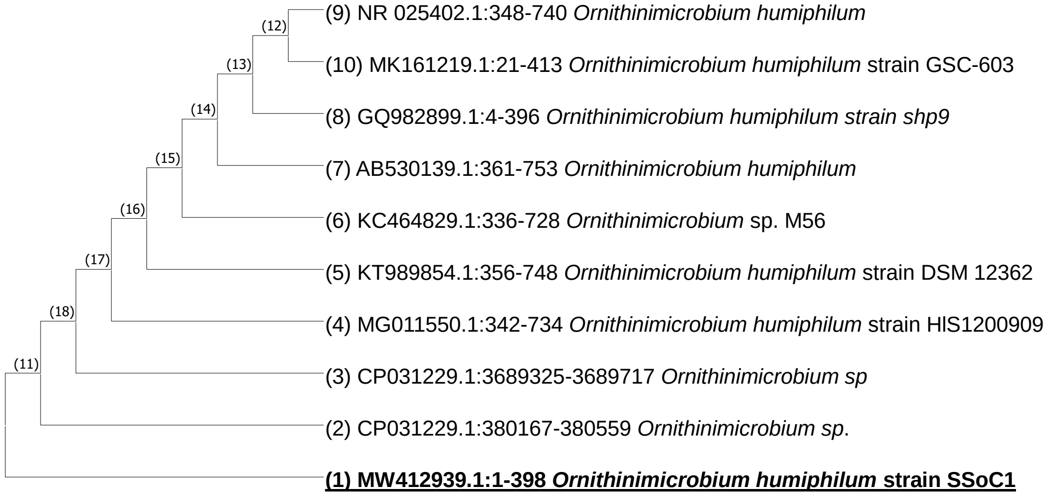

Fig 1.0: Phylogenetic analysis of an isolate SSoC1 identified as *Ornithinimicrobium humiphilum* (Accession Number: MW412939).

Phylogenetic analysis based on 16S rRNA gene sequencing confirmed that isolate SSoC1 belongs to *Ornithinimicrobium humiphilum*. The partial 16S rRNA gene sequence (GenBank accession number: MW412939) showed more than 97% sequence similarity with reference strains of *O. humiphilum* (CP031229, MG011550, KT989854). In the phylogenetic tree, isolate SSoC1 clustered within the clade of *Ornithinimicrobium humiphilum*, thereby confirming its taxonomic identity as *O. humiphilum* strain SSoC1.

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, 0.28 U mL^{-}1, was found at 20^{0}C (Fig. 1.1). Incubation temperature above or below 20^{0}C resulted in a steady decrease in enzyme activity. The findings showed that a large amount of enzyme produced at temperatures between 15^{0}C and 25^{0}C.

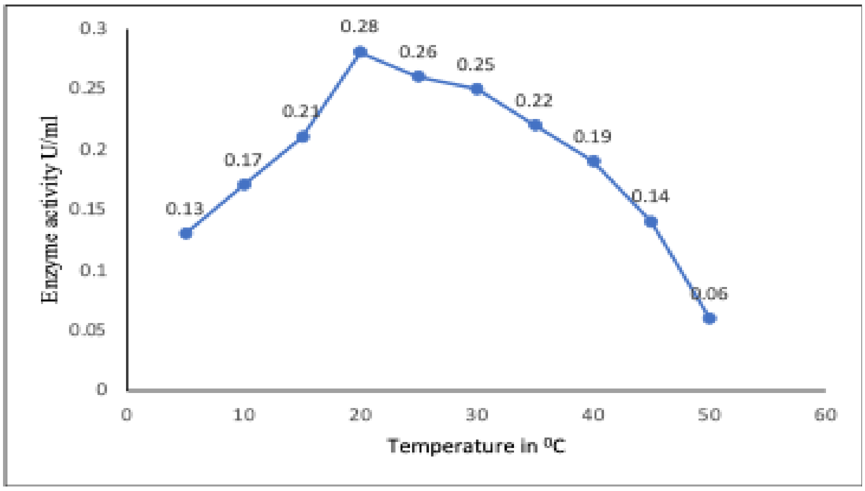

Fig: 1.1: Enzyme activity of *Ornithinimicrobium humiphilum* at different Temperature.

At 20^0C cells were incubated at pH ranging from 4-9 in protease production medium to evaluate the influence of pH on enzyme production while keeping all other parameters stable. Maximum protease production was observed at 7 pH (Fig 1.2). As a consequence, it's advisable that for industrial purposes, minimal temperature gradient at neutral pH in enzyme production is preferable.

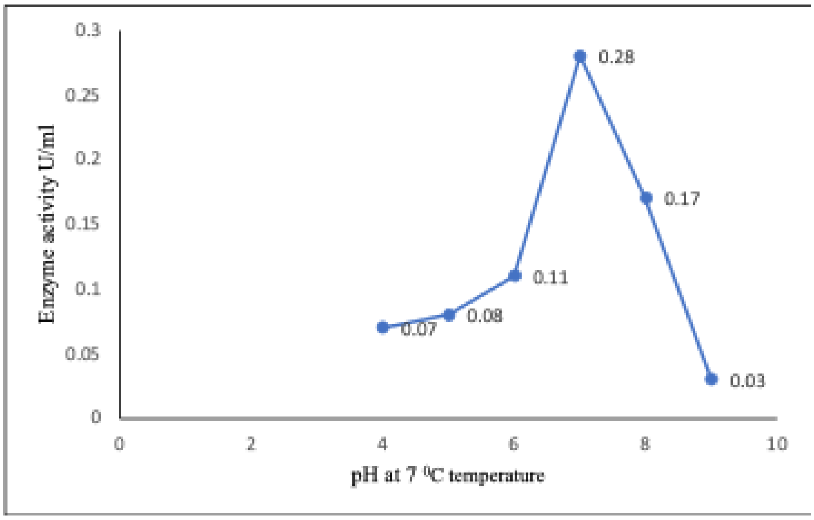

Fig: 1.2: Enzyme activity of *Ornithinimicrobium humiphilum* at different pH at 20^0C temperature.

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Conflict of Interest

The Authors declare no conflicts of interest.

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