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Studies on Proteolytic Psychrotrophic *Stenotrophomonas maltophilia* from Milk and Fermented Milk Products

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Psychrotrophic bacteria are able to multiply in a cold environment (7°C) during refrigerated storage of milk and fermented milk products. They also produce heat resistant exoenzymes such as protease, lipase which degrade protein and fats in milk and milk products and contribute in spoilage of dairy products during refrigerated storage. Ten dairies from Nashik region of Maharashtra in India were assessed for studying the psychrotrophic bacteria present in milk and fermented milk products. During searching of such psychrotrophic bacteria from milk and fermented milk products, one of the isolate was identified as *Stenotrophomonas maltophilia* on the basis of morphological, biochemical, physiological features as well as 16SrRNA sequencing. The base sequence of the isolate was submitted to NCBI GenBank to assign the accession number. The isolate was also analyzed for protease production at refrigeration temperature.The protease enzyme was estimated by qualitative as well as by quantitative methods. Enzyme shows optimum activity at pH of 7 and at 35°C within 10 minutes.

Key words: Psychrotrophs, *Stenotrophomonas maltophilia*, proteolytic, dairy products, Spoilage, 16S rRNA sequence

Milk and dairy products are generally rich in nutrients which provide an ideal growth environment for many microorganisms. Milk is sterile at secretion in the udder but it is contaminated by microorganisms during milking, handling, storage and other pre-processing activities[1]. Milk becomes contaminated with various types of microorganisms which originate from the soil, water or skin and the hair of the animals, utensils or from the milk handlers[2]. After collection of milk, it is preserved at refrigeration temperature. The collection and refrigerated storage of raw milk favored the growth of psychrotrophic bacteria which are able to grow below 7°C, regardless of their optimal growth temperature[3]. *Stenotrophomonas maltophilia* is an aerobic Gram negative nonfermentative bacillus which produces yellow colonies on milk agar. The colonies may turn to dark brown colour with age[4]. Initially classified as *Pseudomonas maltophilia*. *Stenotrophomonas maltophilia* was also grouped in the genus *Xanthomonas* before eventually becoming the type species of Genus *Stenotrophomonas*. *Stenotrophomonas maltophilia* is ubiquitous in aquatic environment, soil and plants, including water, urine or respiratory secretions and is commonly isolated from clinical

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materials, nosocomial infections and frozen foods[4]. It is an uncommon pathogen in humans[5].

During these studies psychrotrophic bacteria belonging to Genus *Stenotrophomonas* was identified and studied for their proteolytic activity.

MATERIAL AND METHODS

Forty different samples of milk and milk products constituting 10 samples of pasteurized milk, 5 samples of flavored milk, 5 samples of curd, 9 samples of shrikhand, 9 samples of lassi and 2 samples of butter were collected from ten different dairies of Nasik region were collected in icebox and brought to the laboratory for further studies and stored at refrigeration temperature.

Isolation and identification

Isolation of psychrotrophs was carried out by streak plate technique using sterile milk agar plates. Plates were incubated at 7°C for 10 days. Colonies developed on milk agar were picked up, purified repeatedly and preserved on nutrient agar slants and fresh transfers were given after every two months. Various isolates obtained were appropriately coded and studied for different morphological, biochemical and physiological characteristics features such as colony characters on milk agar, Gram nature (Hucker and Conn method, 1923), motility by hanging drop method, enzymatic activities such as protease, lipase, oxidase, catalase, amylase, gelatinase, urease, lecithinase, phenylalanine deaminase, arginine hydrolysis and nitrate reduction test. Other tests include Hugh-Leifson's test i.e. oxidation-fermentation test, IMViC test and sugar fermentation test.[6]

These isolates were further identified using 16S rRNA sequencing in which isolation of genomic DNA was carried out using Prepman Ultra sample preparation reagent (Applied Biosystems, Applera, USA). The Microseq 16S rRNA gene kit (Applied Biosystems Division) was used for PCR and sequencing. The sequence generated through automated sequencing was used to search for homologous sequences in the NCBI database (http://www.ncbi.nlm.nih.gov) with the help of BLAST database search tool. These sequences were then submitted to NCBI GenBank to receive the accession number.

Enzyme assay

Protease assay was carried out qualitatively as well as quantitatively

The proteolytic activity was detected by observing a clear zone of hydrolysis around the colonies on skim milk agar after incubation at 7°C for 10 days[7]. Quantitatively, proteolytic activity was determined by modified Folin method[8]. Protease activity in culture supernatants *Stenotrophomonas maltophilia* of grown in 1 liter of liquid yeast extract casein medium was observed after 7°C for 10 days. Protease activities were measured by the amount of acid-soluble fragments released from casein at 7°C. Culture supernatants (100 µl) were incubated with 500 µl of 1% casein and 400 µl of 80mM imidazole-HCl buffer (pH 7.8).After incubation, an equal volume of TCA solution (0.11M trichloroacetic acid, 0.22M sodium acetate and 0.12M acetic acid) was added to the reaction mixture.TCA insoluble materials were removed by centrifugation and the quantity of tyrosine in the cleared supernatant was measured by a modified Folin method. One unit of enzyme activity was defined as the amount of enzyme that released one µg tyrosine mL^{-1} min^{-1} from casein. Protein concentration was estimated by the Biuret method with bovine serum albumin (BSA) as standard.

Effect of temperature on the activity of protease was determined using the substrate as casein. Enzyme activity at various temperatures were measured by incubating the reaction mixtures in 80mM imidazole-HCl buffer (pH 7.8) at 0°,10°,20°,25°,30°,35° and 40°C for 10,20,30 and 40 minutes[9].

Effect of pH on enzyme activity

Enzyme activity at various pH values was observed at 30° C for 10 min in reaction mixture with 80mM buffer at various pHs using phosphate buffer-pH 6, 7, 8: citrate buffer pH 4, 5: trace amino methane HCl buffer pH 9[9].

RESULTS AND DISCUSSION

Colony characteristics, morphology, Gram nature and motility of the isolate is presented in Table 1.

The isolate was Gram negative motile rod shaped bacteria. It can be seen from Table1 that the isolate produced 1mm size yellow opaque

Table 1. Colony characteristics, Gram nature and motility of the isolate

S.No.	Code	Size	Shape	Colour	Margin	Elevation	Opacity
1	WVM(1)	1mm	Circular	yellow	Entire	Convex	opaque

Consistency	Gram nature	Motility
mucoid	Gram negative rods	Motile

Table 2. Biochemical characteristics of the isolate

S.No.	Characteristic	WVM(1)
Enzymatic tests		
1	Catalase	+
2	Oxidase	–
3	Lecithinase	–
4	Amylase	–
5	Gelatinase	+
6	Protease	+
7	Lipase	+
8	Urease	–
9	O/F test	O
10	Nitrate reduction	–
11	Arginine hydrolysis	–
12	Phenylalanine deaminaton	–
IMViC test		
13	Indol production	–
14	Methyl Red	–
15	V.P.test	–
16	Citrate utilization	+
Sugar fermentation tests		
17	Glucose	–
18	Lactose	–
19	Mannitol	–
20	Arabinose	–
21	Xylose	–
22	Galactose	–

O/F=Oxidation/fermentation; A=Acid production; + = positive; - = negative

colonies, circular in shape with entire margin, convex elevation with mucoid consistency.

On the basis of following morphological, biochemical and physiological characteristics [4] the isolate was found to belong to Genus *Stenotrophomonas* and was further identified on the basis of 16S rRNA sequencing as *Stenotrophomonas maltophilia*.

It can be seen from Table 2 the isolate was positive for catalase, gelatinase, protease and lipase enzymes but showed negative results for oxidase, lecithinase, urease, amylase, nitrate reduction, arginine hydrolysis and phenylalanine deaminaton test. The isolate was able to utilize Na citrate as carbon source. Remaining three tests i.e.Indol production, methyl red test and V.P.test were negative. The isolate does not ferment glucose, lactose, mannitol, arabinose, xylose and galactose sugars.

Table 3 represents the identification of bacterial isolate. On the basis of morphological, cultural, physiological characterization as well as 16S rRNA sequencing the isolate WVM (1) was identified as *Stenotrophomonas maltophilia*.

It can be seen from Table 4 when the culture of identified *Stenotrophomonas maltophilia* was inoculated on skim milk agar for determination of proteolytic activity, the diameter of zone of hydrolysis was 6mm and enzyme activity 200 µg/ml.

From Graph (1) and Graph (2) Enzyme shows maximum activity at pH of 7 and at 35°C within 10 minutes. Similarly low optimum temperatures are observed with other protease from psychrophiles and psychrotrophs. An alkaline

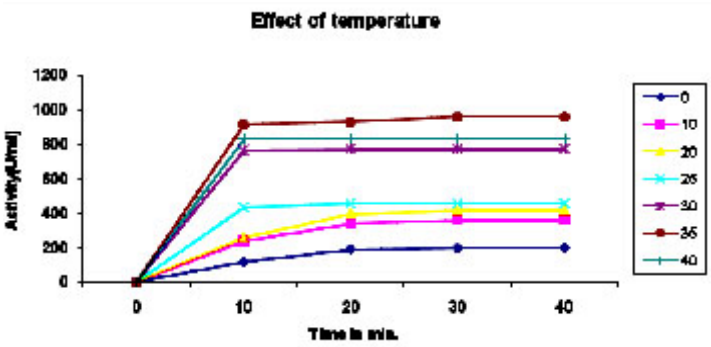
Table 3. Identification of the isolate by 16S rRNA sequencing

Code no.	Identified bacteria	GenBank Accession Number	% Identity
WVM(1)	*Stenotrophomonas maltophilia*	GQ 982892	100%

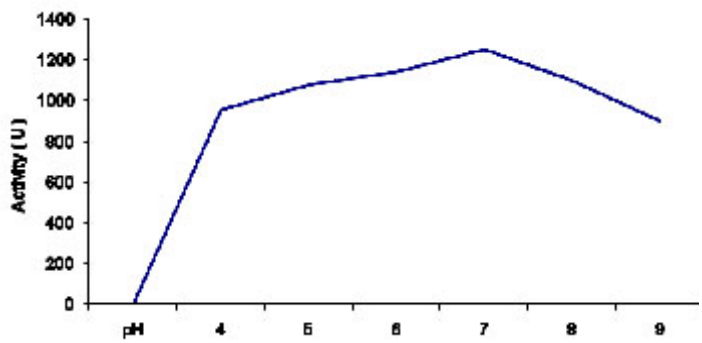
Table 4. Proteolytic activity of *Stenotrophomonas maltophilia* was determined by qualitative and quantitative methods

CodeNo.	Name of Isolate	Proteolytic activity	
	.	Plate assayZone diameter(mm)	Enzyme activity µg mL^{-1}
WVM(1)	*Stenotrophomonas maltophilia*	6	200

protease from a psychrophile, *Escherichia freundii*, had an optimum temperature of 25°C at pH 10, which is slightly lower than that of the protease of *Pseudomonas fluorescens* 114 (35°C at pH 6.5 and 10)[10]. However, yeast isolated in Antartica, *Candida humicola*, produced a protease with an optimum temperature of 37°C[11] and a psychrophile, *Xanthomonas maltophilia*, produced a protease with an optimum temperature of 50°C[12].



Graph 1. Effect of temperature on the activity of the protease



Graph 2. Effect of pH on the activity of the protease

CONCLUSION

Psychrotrophic *Stenotrophomonas maltophilia* was isolated from milk and fermented milk products exhibiting 100% similarity at the 16S rRNA level. *Stenotrophomonas maltophilia* produced protease enzyme and hydrolyzed milk proteins at refrigeration temperature (7°C) resulting in to reducing the shelf-life of milk and milk products.

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