PROTEOLYTIC PSYCHROTROPHIC *Bacillus cereus* FROM MILK AND FERMENTED MILK PRODUCTS

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ABSTRACT

Milk and fermented milk products were collected from ten different dairies of Nasik region of Maharashtra in India to study the psychrotrophic bacteria present in them. Due to high nutritive value, milk is an excellent culture medium for a variety of microorganisms and being a perishable type of food, gets easily spoiled by the activity of microorganisms present in it. Milk and milk products are generally preserved at refrigeration temperature (4-7^{0}C). Psychrotrophic bacteria are able to multiply under this temperature. During the study of these psychrotrophic bacteria present in milk and fermented milk products , one of the isolate was found to be *Bacillus cereus* which was identified on the basis of morphological, biochemical, physiological features as well as 16S rRNA sequencing. *Bacillus cereus* showed proteolytic activity which was estimated qualitatively by using skim milk agar and quantitatively by Folin-Lowery methods. Protease enzyme produced by *Bacillus cereus* showed maximum activity at 40^{0}C and at pH 7.

Key Words: Psychrotrophs, Proteolytic, Dairy products, 16S rRNA sequence, Fermented.

INTRODUCTION

The normal preservation method used in the dairy industry is refrigeration for the storage of milk and milk products. At this refrigeration temperature, milk and milk products allows the growth of psychrotrophic bacteria. Their metabolic activities causes chemical deterioration of milk and milk products resulting in various types of undesirable changes like off flavors, colour, rancidity, ropiness etc. During storage under low temperature, the milk undergoes spoilage due to proteinases and lipases released by psychrotrophic bacteria[1]. The psychrotrophs causes spoilage of milk by producing heat resistant proteolytic enzymes which induce degradation of casein[2]. These undesirable changes adversely affect the economics of dairy industry.

Bacillus cereus is a Gram positive, spore forming, motile rods, commonly found in soil, plant material, hay, raw and processed food[3]. This organism also frequently found in pasteurized milk, causing spoilage due to production of lipases and protease[4]. The bacterium is responsible for two different types of food borne diseases- the emetic syndrome caused by ingestion of a preformed toxin in the food and the diarrheal syndrome caused by a different toxin that can be formed in the food but also in small intestine[5]. These organisms survive under heat treatments used for processing of the products, activates spore germination and resulting in spoilage of products. The organism is associated with defects such as off flavors; sweet curdling, bitty cream caused by proteinase, lipase and phospholipase enzymes affecting the shelflife of milk and heat treated dairy products[6]. Dried milk products are frequently contaminated with *B.cereus*, principally with its spores[7,8]. Viable spores of *B.cereus* may germinate and the vegetative cells can proliferate and produce toxin, which could potentially even occur at

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refrigeration temperatures[6]. The psychrotrophic variants of *B.cereus* have the capacity to grow and generate toxin at storage temperatures above 6^0C[4,9]. In particular, the prevalence of *B.cereus* reported in dried milk products ranged from 10% to 100%, reaching levels from 0.3 to 10^3 cells or spores g^{-1} [8,10]. For milk powder, an incidence of *B.cereus* 15-75% has been reported, with counts that ranged from 5 to 10^3 cfu g^{-1}, [11,12]. An incidence of *B.cereus* was 54% for samples of infant food, with counts ranged from 0.3 to 600 cells g^{-1}. Raw and pasteurized milk as well as refrigerated food frequently harbor psychrotrophic strains of *B.cereus*[11,13].

During the study of psychrotrophic bacteria, species of *Bacillus cereus* was identified and studied for their proteolytic activity.

MATERIAL AND METHODS

Milk, flavored milk and fermented 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 of Maharashtra in India 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 7^0C 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, motility by hanging drop method, spore staining by Dorner's 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 tests[14]. Effect of temperature, pH and salt concentration on growth of isolates were studied as

Effect of temperature: The isolates were inoculated in sterile nutrient broth and incubated at different temperatures such as 7^0C for 10 days, 15^0C, 25^0C, 37^0C, 45^0C and 55^0C for 24h. After incubation tubes were observed for growth.

Effect of pH: The isolates were inoculated in sterile nutrient broth having different pH values such as 4, 5, 6, 7, 8, 9 and 10 and incubated at 7^0C for 10 days. After incubation tubes were observed for growth.

Effect of salt concentration: The growth of isolates at different concentration of NaCl was studied. The nutrient broths having different salt concentration were used such as 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7 % respectively. After incubation at 7^0C for 10 days tubes were observed for growth.

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 with the help of BLAST database search tool. These sequences were then submitted to NCBI GenBank to receive the accession number. A phylogenetic tree was generated by the neighbor-joining method using MEGA4[15].

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 7^0C for 10 days[16]. Quantitatively, proteolytic activity was determined by modified Folin method[17]. Protease activity in culture supernatants of *B. cereus* grown in 1 liter of liquid yeast extract casein medium was observed after 7^0C for 10 days. Protease activities were measured by the amount of acid-soluble fragments released from casein at 7^0C. Culture supernatants (100 µl) were incubated with 500 µl of 1% casein and 400 µl of 80 mm imidazole-

HCl buffer (pH 7.8).After incubation, an equal volume of TCA solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.12 M 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[17]. 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 as standard.

Effect of temperature : The activity of protease was determined using the substrate as casein. Enzyme activity at various temperatures were measured by incubating the reaction mixtures in 80 mm imidazole-HCl buffer (pH 7.8) at 7^0, 15^0, 20^0, 25^0, 30^0, 35^0, 40^0, 45^0 and 50^0C for 10 min[18].

Effect of pH on enzyme activity: Enzyme activity at various pH values was observed at 7^0 C for 30 min. in reaction mixture with 80 mm buffer at various pH using phosphate buffer pH 6, 7, 8 ; citrate buffer pH 4, 5 and trace amino methane HCl buffer pH 9[18].

RESULTS AND DISCUSSION

Colony characteristics, morphology, Gram nature and motility of the isolate is presented in **Table 1** The isolate was Gram positive motile rod shaped bacteria. It can be seen from **Table 1** that the isolate produced 1 mm size white opaque colonies, circular in shape with entire margin, convex elevation with smooth consistency.

It can be seen from **Table 2** the isolate was spore bearing and positive for catalase, gelatinase, protease, amylase, lecithinase and nitrate reduction test but showed negative results for oxidase, urease, lipase, phenylalanine deamination and arginine hydrolysis. The isolate ferments glucose, sucrose, galactose and trehalose with production of acid while does not ferment lactose, mannitol, xylose, maltose, sorbitol and mannose . The tests for Indole production and citrate utilization were negative while methyl red and Voges-Proskauer's test were positive.

Table 2 : Biochemical characteristics of the isolates

Characteristic	Code of isolate SNM-1
Spore Staining (Dorner's Method)	+
Enzymatic Test	
Catalase, Amylase, Gelatinase, Protease, Lecithinase	+
Oxidase, Lipase, Urease	-
Nitrate reduction test	+
Phenylalanine Deamination, Arginine hydrolysis	-
O/F test	F
Sugar Fermentation Test	
Glucose, Sucrose, Galactose, Trehalose	A
Lactose, Mannitol, Xylose, Maltose, Sorbitol, Mannose	-
IMViC Test	
Indole Production, Citrate Utilization	-
Methyl Red, Voges-Proskauer's test	+

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

The results of the growth of isolate at various temperatures are presented in **Table 3**.

It can be observed that the isolate showed growth at 7^0C, 15^0C, 25^0C, 37^0C and 45^0C but no growth was observed at 55^0C. Maximum growth of the

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

Code of isolate	Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram Nature	Motility
SNM-1	1 mm	Circular	white	Entire	Convex	opaque	Smooth	Gram Positive rods	Motile

Table 3: Effect of temperature on growth of the isolate

Code of isolate	Temperature					
	7°C	15°C	25°C	37°C	45°C	55°C
SNM-1	+	+	++	+++	+	-

+ Growth ++ : Moderate growth +++ : Maximum growth - : No growth

Table 4 represents the results of growth of isolate at different pH values. The isolate grew at pH 6, 7, 8 and 9 while maximum growth of the isolate was observed at pH 7 but no growth was observed at pH 4, 5 and 10.

Table 4 : Effect of pH on growth of the isolate

Code of isolate	pH						
	4	5	6	7	8	9	10
SNM-1	-	-	+	+++	+	+	-

+ : Growth ++ : Moderate growth +++ : Maximum growth : No growth

Table. 5 represents effect of salt concentration on growth of the isolate. It can be observed that the isolate tolerated upto maximum 4% salt concentration and did not grow at 4.5%, 5%, 5.5%, 6% , 6.5% and 7% salt concentration respectively.

Table 5 : Effect of salt concentration on growth of the isolate

Code of isolate	Salt concentration (%)													
	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7
SNM-1	+++	++	+	+	+	+	+	+	-	-	-	-	-	-

+ : Growth ++ : Moderate growth +++ : Maximum growth : No growth

Table 6 represents the identification of bacterial isolate. On the basis of morphological, cultural, physiological characterization[19] as well as 16S rRNA sequencing the isolate SNM-1 was identified as *Bacillus cereus* strain shp 15.

It can be seen from **Table. 7** when the identified culture of *B. cereus* was inoculated on skim milk agar for determination of proteolytic activity, the zone diameter of hydrolysis was 7 mm and enzyme activity was found to be 169 µg/ml.

Table 6 : Identification of the isolate by 16S rRNA sequencing

Code of isolate	Identified bacteria	GenBank Accession Number	% Identity
SNM-1	*Bacillus cereus* strain shp 15	JN 230858	99%

Table 7 : Proteolytic activity of Bacillus cereus was determined by qualitative and quantitative methods.

Code of isolate	Name of Isolate	Proteolytic activity	
		Plate assay Zone diameter(mm)	Enzyme activity (µg/ ml)
SNM-1	*Bacillus cereus* strain shp 15	7	169

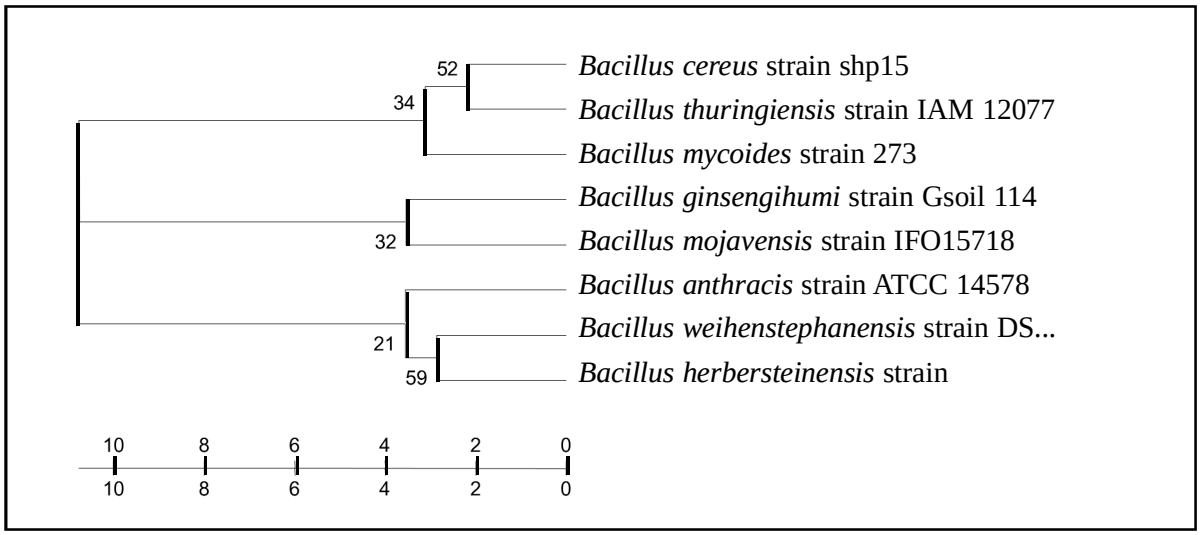

Fig. 1 : Phylogenetic tree of isolates belonging to the genus *Bacillus*.

Identification of the bacterial strain as Bacillus cereus was also done by 16S rRNA gene sequence analysis and the nucleotide sequence was submitted to the gene bank under the accession number JN 230858. Phylogenetic position of Bacillus cereus strain shp15 in relation to the species of this genus found in the Gene Bank database is as illustrated **(Fig.1)**.

Fig. 2 and **Fig. 3** Shows on enzyme activity based on relative enzyme activity values. The maximum activity of the enzyme was taken as % 100.

From **Fig. 2** and **Fig. 3** enzyme shows maximum activity at pH of 7 and at 40^0C. Although proteolysis was highest at 40^0C, considerable activity was observed at refrigeration temperature. Similarly low optimum temperatures are observed with other protease from psychrophiles and psychrotrophs. Certain psychrotrophic *B.cereus* strains can grow in foods at temperature as low as 4-6^0C and may produce the diarrheal type enterotoxin[4,6]. 92% of the *Bacillus* isolates were found to have proteolytic and or lipolytic activity[20].

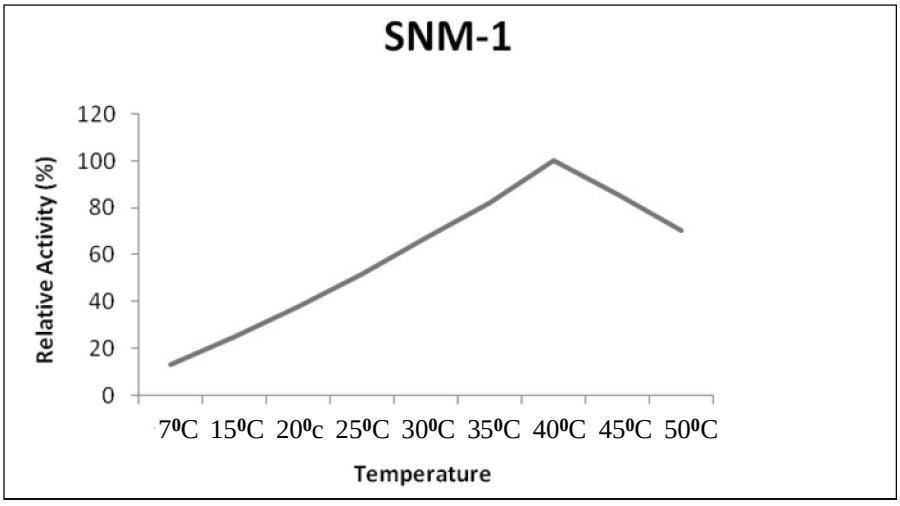

Fig. 2 : Effect of temperature on the activity of the protease.

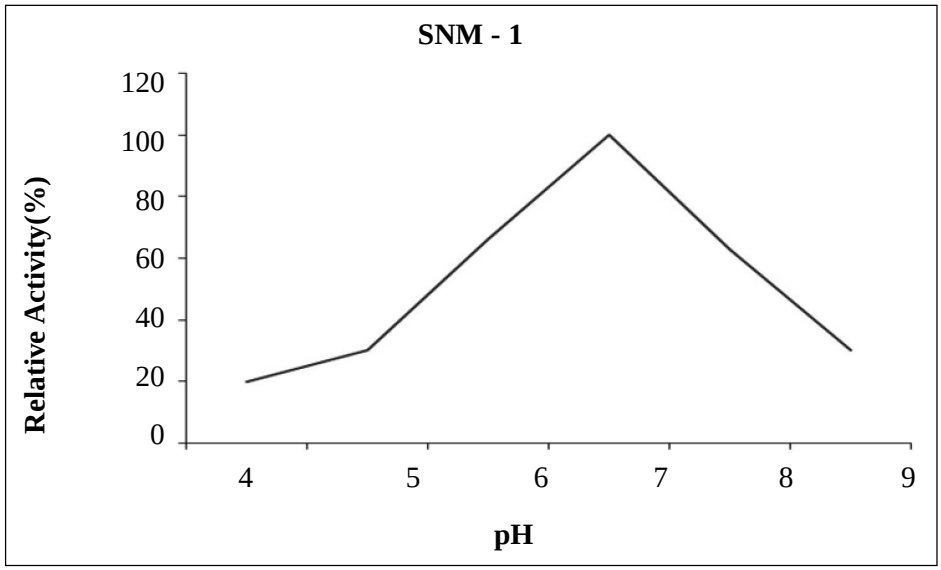

Fig. 3 : Effect of pH on the activity of the protease.

CONCLUSION

Psychrotrophic *Bacillus cereus* was isolated from milk and fermented milk products exhibiting 99% similarity at the 16S rRNA level. *Bacillus cereus* produced protease enzyme and hydrolyzed milk proteins at refrigeration temperature (7^0C) resulting in to reducing the shelflife of milk and milk products. Protease enzyme produced by *Bacillus cereus* showed maximum activity at 40^0C and at pH 7.

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