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Studies on Proteolytic and Lipolytic Psychrotrophic *Pseudomonas* species in Milk and Fermented Milk Products

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Psychrotrophic bacteria are capable of surviving in a cold environment (4°-7°C) regardless of their optimum growth temperature. They can be found in soil, deep sea water and in foods. The psychrotrophic bacteria isolated from dairy products can grow at refrigeration temperature and their number increases during cold storage and makes the food worse by producing exoenzymes such as protease, lipase etc.For searching of these psychrotrophs, milk and fermented milk products were collected from ten different dairies of Nasik region in Maharashtra and analyzed for total psychrotrophic count. Two psychrotrophic bacterial species that were present in milk and fermented milk products belongs to Genus *Pseudomonas*. These species of *Pseudomonas* were further identified as *Pseudomonas putida* and *Pseudomonas stutzeri* on the basis of morphological, biochemical and physiological features as well as 16S rRNA sequencing. The base sequences of these isolates were submitted to NCBI GenBank to assign the accession number. They were also analyzed for protease and lipase production at refrigeration temperature. *Pseudomonas putida* showed both proteolytic as well as lipolytic activity whereas *Pseudomonas stutzeri* showed only lipolytic activity. Both the enzymes were estimated by qualitative as well as by quantitative methods.

Key words: Psychrotrophs, *Pseudomonas*, lipolytic, proteolytic, 16S rRNA sequence.

Bacteria in the family Pseudomonadaceae are among the most important spoilage causing bacteria originating in refrigerated raw milk[1]. They were considered as psychrotrophs growing well at common refrigeration temperature (0°C – 15°C)[1]. *Pseudomonas* species are the most common organisms in raw or pasteurized milk at the time of the spoilage[2].

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Different species of *Pseudomonas* which are involved in spoilage of milk and milk products includes *Pseudomonas fluroscence, P. putida, P. fragi* and *P. putrefaeciens*, less frequently *P. aeruginosa*[3]. *P. fluroscence* produces heat stable lipases and proteases [(4)] which are responsible for milk defects such as bitterness, rancidity, fruity and cardboardy flavour, casein breakdown and ropiness due to production of slime and coagulation of proteins[1,4,5].

These species are able to grow to high numbers during refrigerated storage and also produces heatstable extracellular lipases, protease and lecithinases which can further contribute to milk spoilage. *Pseudomonas* species synthesizes heat stable enzymes at refrigeration temperature, mainly at the end of the log cell growth stage[2].

Water and soil are primary source of *Pseudomonas* species[1, 4]. Degradation of milk and milk products due to enzymatic activities can reduce the shelf life of milk and milk products. *Pseudomonas* in particular have been shown on several occasions to be responsible for defects in raw milk, pasteurized milk and milk products[6]. Cox and MacRae[7] identified two phenotypes , comprising approximately 30% and 5% of the strains, as *Pseudomonas fluroscence* and *Pseudomonas fragi* respectively. Bergtsson *et al* .,[8] observed evidence of ongoing proteolysis during storage of sterile milk which had supported *Pseudomonas* growth prior to sterilization.

During these studies psychrotrophic bacteria belonging to Genus *Pseudomonas* was identified and studied for their proteolytic and lipolytic 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 & 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[9].

Effect of temperature on growth of the isolates

The isolates were inoculated in sterile nutrient broth and incubated at different temperatures such as 7°C for 10 days, 15°C, 25°C, 37°C and 55°C for 24hrs to 48hrs. After incubation tubes were observed for growth.

Effect of pH on growth of the isolates

The same 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°C for 10 days. After incubation tubes were observed for growth.

Effect of salt concentration on growth of the isolates

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% and 6% respectively. After incubation at 7°C 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 (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 and lipase assay were 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[10]. Protease activity in culture supernatants of *Pseudomonas putida* grown in 1 liter of liquid LB 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[11]. 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.

The lipolytic activities of the isolates were tested by using tributyrin agar after incubation at 7°C for 10 days. Presence of clear zone of hydrolysis was considered as lipolytic bacteria. Quantitatively, lipase activity was assayed by titrimetric method of Marcin *et al.,* (1993) at 7°C using 0.1 N NaOH to neutralize the fatty acids that are produced after breakdown of tributyrin. One unit of lipase was defined as the amount of enzyme required to release 1µmol of fatty acids (in terms of acetic acid) per min. under assay conditions.

RESULTS AND DISCUSSION

Colony characteristics, morphology, Gram nature and motility of the isolates are presented in Table 1.

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

S.No.	Code	Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram nature	Motility
1	WAM(1)	1mm	Circular	Colourless	Entire	Convex	Semitransparent	Mucoid	Gram negative rods	Motile
2	SPM(1)	1mm	Circular	Colourless	Entire	Convex	Semitransparent	Mucoid	Gram negative rods	Motile

Both the isolates were Gram negative motile rods. It can be seen from Table 1 that the isolates produced 1mm size colourless semitransparent colonies, circular in shape with entire margin, convex elevation with mucoid consistency.

On the basis of following morphological, biochemical and physiological characteristics[13] two isolates were found to belong to Genus *Pseudomonas* and were further identified on the basis of 16S rRNA sequencing as two different species of *Pseudomonas*.

It can be seen from Table 2 both the isolates were positive for catalase, oxidase and lipase enzymes but they showed negative results for lecithinase, urease,amylase,gelatinase,nitrate reduction,arginine hydrolysis and phenylalanine deaminaton test. Only WAM (1) produces protease enzyme while SPM (1) was unable to produce it. Both the isolates were able to utilize Na citrate as carbon source. WAM (1) showed Methyl red test positive. Remaining two tests i.e.Indol production and V.P.test were negative. Both the isolates produced acid from arabinose sugar and only WAM (1) produces acid from

Table 2. Biochemical characteristics of the isolates

S.No.	Characteristic	WAM(1)	SPM(1)
	Enzymatic tests		
1	Catalase	+	+
2	Oxidase	+	+
3	Lecithinase	–	–
4	Amylase	–	–
5	Gelatinase	–	–
6	Protease	+	–
7	Lipase	+	+
8	Urease	–	–
9	O/F test	O	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	A	–
18	Lactose	–	–
19	Mannitol	–	–
20	Arabinose	A	A
21	Xylose	–	–
22	Galactose	–	–

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

glucose. None of the isolates utilized lactose, mannitol, xylose and galactose.

The results of the growth of isolates at various temperatures were presented in Table 3. It can be observed that both the isolates showed growth at 7°C,15°C,25°C and 37°C but no growth was observed at 55°C. Maximum growth of the isolates was observed at temperature between 15°C to 25°C.

Table 3. Effect of temperature on growth of the isolates.

S. No	Bacteria Temperature	7°C	15°C	25°C	37°C	55°C
1	WAM (1)	++	+++	+++	+	_
2	SPM (1)	++	+++	+++	+	_

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

Table 4 presents the results of growth of isolates at different pH values. It can be seen from the table both the isolates grew at pH 6, 7, 8 and 9. At pH 5 only SPM (1) was showed its growth and at pH 10 only WAM (1) was abled to grow while maximum growth of both the isolates was observed at pH 7. None of them were abled to grow at pH 4.

Table 4. Effect of pH on growth of the isolates

S.No	Bacteria pH	4	5	6	7	8	9	10
1	WAM (1)	-	-	+	+++	++	+	+
2	SPM (1)	-	+	++	+++	++	+	-

Table 5 represents effect of salt concentration on growth of the isolates. It can be observed that both the isolates tolerated upto maximum 3% salt concentration and did not grow at 3.5, 4, 4.5, 5, 5.5 and 6% salt concentration respectively.

Table 5. Effect of salt concentration on growth of the isolates

S. No.	Bacteria	Salt concentration (%)											
		0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6
1	WAM (1)	+++	++	++	+	+	+	-	-	-	-	-	-
2	SPM (1)	++	+++	++	+	+	+	-	-	-	-	-	-

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

Table 6 represents the identification of bacterial isolates. On the basis of morphological, cultural, physiological characterization as well as 16S rRNA sequencing the isolates WAM (1) and SPM (1) were identified as *Pseudomonas putida* and *Pseudomonas stutzeri* respectively.

As shown in Table 7 and Fig. 1 when the cultures of these two identified *Pseudomonas* species were inoculated on skim milk agar for determination of proteolyic activity, the diameter of zone of hydrolysis 6mm for *Pseudomonas putida* and enzyme activity was 300 µg /ml.

Table 6. Identification of the isolates by 16S rRNA sequencing.

Isolate No.	Identified bacteria	GenBank Accession	% Identity Number
1	*Pseudomonas putida*	GQ 982897	99%
2	*Pseudomonas stutzeri*	GQ 982903	99%

Table 7. Proteolytic and Lipolytic activities of *Pseudomonas* species were determined by qualitative and quantitative methods

S. No.	Name of Isolate	Proteolytic activity		Lipolytic activity	
		Plate assay Zone diameter(mm)	Enzyme activity ìg mL^{-1}	Plate assay Zone diameter(mm)	Enzyme activity Units mL^{-1}
1	*Pseudomonas putida*	6	300	6	0.018
2	*Pseudomonas stutzeri*	_	_	5	0.014

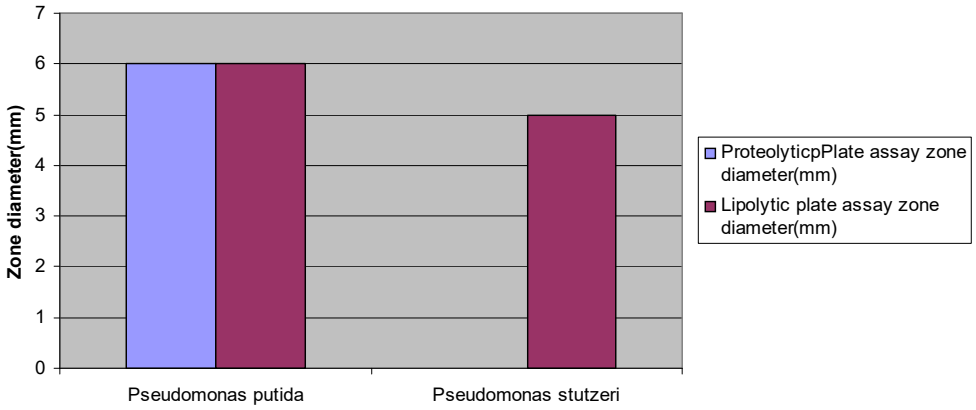

Fig. 1. Enzyme activity

Results of lipolytic activity showed that the diameter of zone of hydrolysis on tributyrin agar by *Pseudomonas putida* was 6 mm and enzyme activity 0.018 Units/ml which was greater as compared to lipolytic activity shown by *P. stutzeri.*

CONCLUSION

Two psychrotrophic species of *Pseudomonas* were isolated from milk and fermented milk products exhibiting maximum similarity (99%) at the 16S rRNA level with *Pseudomonas putida* and *P. stutzeri.* Clear zone formation was observed around the colonies of *P. putida* and *P. stutzeri* when grown at 7°C on tributyrin agar plates, indicating that these lipolytic psychrotrophs were present in milk and fermented milk products in India. *P. stutzeri* were positive only for lipase but negative for protease enzyme. On the other hand *P. putida* produced protease as well as lipase enzyme at refrigeration temperature (7°C). Lipolytic activity shown by *P. putida* was more as compared to *P. stutzeri*. These enzymes produced by *Pseudomonas* species may reduce the shelf-life of milk and milk products.

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