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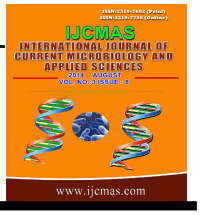

Original Research Article

Isolation and characterization of bacteria with spoilage potential from some refrigerated foods of West Bengal, India

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A B S T R A C T

Storage at low temperature is a popular method for preservation of foods containing heat labile nutrients including vitamins. But improper handling and temperature abuse during transit and storage of these foods may favour growth of microflora initially present in the raw materials. Production of extracellular enzymes by these microorganisms at different temperature indicates their spoilage potentiality. This paper deals with the enumeration and characterization of bacteria with spoilage potential from some refrigerated foods viz. butter, curd, fruit juice and pasteurized milk. All together 100%, 28.6% and 53.6% of the isolates studied produced amylase, caseinase and lipase, respectively. Four of the isolates two each from butter and curd produced all the three types of enzymes. More over 92.8% of the isolates were multiple drug resistant. The study indicates that most of the organisms were gram positive and all were psychrotrophic with spoilage potential producing at least one of the three spoilage causing enzymes.

Keywords

Refrigerated foods, butter, curd, fruit juice, extracellular enzymes, drug resistance

Introduction

A food is considered spoiled when the food is not acceptable for human consumption. Acceptance for consumption and organoleptic qualities of foods (colour, texture, flavor, shape etc.) being subjective varies with culture and socioeconomic condition, so the parameters to judge spoilage also change. However, during spoilage, food nutrients principally carbohydrates, proteins, lipids and other non-protein nitrogenous substances are degraded by the catalytic actions of different enzymes produced either by the food ingredients itself or microorganisms infesting the foods. Food spoilage is an area of global concern as it not only causes economic loss and diminishes nutritional value, but also reduces the amount of foods safe for human consumption. To reduce spoilage, a number of preservation techniques have been formulated primarily based on manipulation of the factors affecting microbial metabolism. One of such factors is low temperature or its combination with other factors. Low temperature storage of food includes chilling and freezing. As the storage temperature is lowered, the rate of chemical reaction causing spoilage is

reduced increasing the shelf life of food products. Chilling is done by storing foods at temperature near, but above their freezing point, typically 0°C-5°C (Kondratowicz and Matusevičius, 2002). Low temperature storage exert a selective effect preventing the growth of mesophiles and leading to a microflora predominated by psychrotrophs owing to presence of higher levels of unsaturated and short chain fatty acids in their membrane lipids. Though mesophiles cannot grow at chill temperature they are not necessarily killed and may resume growth when temperature abuse during storage occurs. Moreover some pathogenic microorganisms causing gastrointestinal ailments may grow albeit slowly at low temperatures (Adams and Moss, 1995).

Microbial spoilage is caused by enzymes most of which are intracellular and some are extracellular. The enzymes are principally amylases, proteases and lipases. Though catalytic activity of microbial enzymes is reduced at low temperature they may not be inactivated completely. Carbohydrates being the most preferred source of energy, production of extracellular amylases are very important for the growth and nutrition of microorganisms present. Proteolysis causes off-flavour. Lipases are water soluble ubiquitous enzymes produced from plants, animals, fungi and bacteria (Wooley and Petersen, 1994). They are serine hydrolases and contain the consensus sequence G-X_1-S-X_2-G as the catalytic moiety, where G = glycine, S = serine, X_1 = histidine and X_2 = glutamic or aspartic acid (Svendsen, 1994). They catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol and fatty acids (Joseph et al., 2008). Most of the bacterial lipases reported so far are constitutive, non-specific in their substrate specificity and few of these are thermostable (Macrae and Hammond, 1985)

In recent years chilling storage of foods has increased enormously. The reasons behind increasing popularity of chilled foods are multifactorial. Now-a-days consumers demand for fresh foods without hazardous chemical preservatives while at the same time they require convenience of occasional shopping to conserve time.

Though consumption of fruit juices is becoming popular rapidly (Zabidah et al., 2011), reports are there that fruit juices containing phenolics, flavonoids and ascorbic acid, may lose some of their antioxidant molecules during storage in refrigerator (Sarkar et al., 2014). From microbiological point of view good quality raw materials and hygienic handling are key requirements for the production of safe chill foods. Moreover, the availability of an efficient cold chain from manufacture to consumption is important for good chilling preservation that might be lacking in developing countries like India. However, reports regarding microflora with spoilage potential present in refrigerated foods viz. butter, curd, fruit juice and pasteurized milk in India are scanty. Hence the present study was undertaken to isolate and characterize bacteria from marketed refrigerated foods with spoilage potential and preparation of their antibiotic resistance pattern.

Materials and Methods

Collection of samples

The samples of pasteurized milk, curd, packed butter & packed fruit juice stored in refrigerator were collected from different shops of Barrackpore area (Latitude: 22.76°N, Longitude: 88.37°E) of West Bengal, India, in the month of November, 2013. The collected samples were transported to the laboratory aseptically in

an ice box and kept into refrigerator until analyzed.

Isolation of bacteria

Representative samples (10 ml for milk and fruit juice and 10 g for butter and curd) were homogenized with 90 ml sterile physiological saline (0.85% wv^{-1} sodium chloride, pH 7.2) by shaking vigorously at room temperature in a rotary orbital shaker for 2 minute. 0.1 ml of the appropriate dilutions was spread-plated on the surface of a dried Nutrient agar (HiMedia MM012) plate and incubated at 30°C for 72 h. After incubation, the colonies appearing on the suitable plates (30-300 colonies per plate) were counted and expressed as colony forming units (cfu) per ml or per g fresh weight sample. The isolated colonies were classified based on their morphology. Single isolate from each morphotype was selected for further analysis, purified by repeated streaking on nutrient agar and stored on nutrient agar slants at 4°C for future study.

Growth at different temperatures

Each of the isolates was tested for growth on nutrient agar plates at different temperatures viz. 5°C, 15°C, 25°C, 35°C, 45°C and 55°C. Briefly one loopful of 18h old culture in nutrient broth (HiMedia M002) was streaked on the surface of nutrient agar plates and incubated upto 72 h at the selected temperatures. Visible growth in naked eye was monitored daily.

Production of enzymes at different temperature

Isolates showing growth at the selected temperatures in nutrient agar plates were tested for the production of enzymes viz. amylase, caseinase and lipase on starch agar (HiMedia M107S), standard method caseinate agar (HiMedia M588) and tributyrin agar base (HiMedia M157) supplemented with 1.0% vv^{-1} tributyrin (HiMedia FD081) respectively. Briefly one loopful of 18 h old culture in nutrient broth was streaked singly on the surface of respective medium and incubated for 72 h at their previously observed growth temperatures. Amylase production was tested by flooding Lugol's iodine solution on incubated starch agar plates to check formation of clear zones surrounding the colony. Colonies surrounded by clear zones indicated production of caseinase and lipase.

Susceptibility to antibiotics

Antibiotic susceptibility was tested using the disc agar diffusion method (Bauer et al., 1966). Three colonies grown on tryptone soya agar (HiMedia M290) plates at 30°C for 24 h, were transferred to 5 ml tryptone soya broth (HiMedia M011) and incubated at the same temperature for 6 h. A sterile cotton swab was dipped into the inoculum and applied evenly onto Mueller-Hinton agar (HiMedia M173) plate (4 mm thick). After drying for 15 min, various antibiotic susceptibility test discs (HiMedia) were applied aseptically. The plates were incubated at 30°C for 18 h. The zones showing complete inhibition were measured.

Morphological and biochemical characterization

The isolates were morphologically characterized by their Gram reaction and cell morphology. Biochemical characterization included production of indole from tryptophan, mixed acid fermentation from glucose (MR reaction), acetylmethylcarbinol production from glucose (VP reaction), utilization of citrate as sole carbon source, production of ammonia from hydrolysis of arginine and

gelatinase production to hydrolyze gelatin (Harrigan, 1998).

Results and Discussion

Study of the total aerobic mesophilic bacterial count shows that the bacterial load was highest in fruit juice (1.16 x 10^9 cfu ml^{-1}) while lowest in curd (2.08 x 10^8 cfu g-1). Pasteurized milk and butter samples contained 2.68 x 10^8 cfu ml^{-1} and 3.80 x 10^8 cfu g^{-1} total aerobic mesophilic bacteria, respectively. A total of eight, nine, five and six morphotypes of bacterial colony could be recovered from the samples of butter, curd, fruit juice and milk, respectively. Some of the colonies from butter and curd were mucoid in consistency.

Taking one isolate from each morphotype, altogether twenty-eight isolates were selected for study of growth at different temperatures (Table 1). None of these isolates could grow at 5°C and even at 15°C within 24 h of incubation. First visible growth was observed at 15°C after 48 h of incubation. Out of the eight isolates from butter, two could grow at a maximum of 55°C. Three isolates could grow at a maximum of 45°C after 24 h, one after 72 h and rest two could not grow at 45°C at all. Of the nine isolates from curd, three isolates showed growth at 45°C after 24 h of incubation while another three showed growth at 45°C only after 72 h of incubation. Rest three isolates could not grow at 45°C at all. Of the five isolates from fruit juice, one showed growth at 45°C after 72 h of incubation. Rest four from fruit juice and six from milk showed highest growth temperature of 35°C.

Qualitative assay of amylase production (Table 2) shows that all the twenty eight (100%) isolates produced amylase. Nineteen of these barring one from butter, three each from curd and fruit juice and two from milk produced amylase at both 15°C and 25°C. Study of the caseinase production (Table 3) shows that eight of the twenty-eight (28.6%) isolates belonging to butter, curd and milk produced caseinase. These included three each from butter and curd and two from milk. Lipase production analysis (Table 4) shows that fifteen of the twenty-eight (53.6%) isolates produced lipase. These included six from butter and nine from curd. The results of antibiotic susceptibility analysis of the twenty-eight isolates to eleven different antibiotics including three β-lactams, two cationic peptides, four aminoglycosides, one fluorinated derivative of quinolone and one tetra-cyclic hydrocarbon are shown in Table 5. All the isolates were resistant to ampicillin while more than 80% of the isolates were resistant to ticarcillin and colistin. Hundred percent of the isolates were sensitive to imipenem and streptomycin.

All the isolates from milk were gram positive in nature. One isolate each from butter and fruit juice and two from curd were gram negative. Eighteen isolates were rods while ten isolates were cocci. All the isolates were indole and methyl red negative while VP and citrate positive. All the isolates hydrolyzed gelatin but none could hydrolyze arginine.

All the four types of foods in our study had a high mesophilic bacterial count. Heat treatment is done during processing of raw materials to kill the pathogenic and spoilage causing bacteria to increase the shelf-life of final products before packaging. Milk and fruit juice contain plenty of nutrients including heat labile vitamins making these products unsuitable for heat treatment at very high temperature. Moreover high level of bacterial count in the raw materials, discontinuous cold chain transit and storage

of these products at ambient temperature in the retail outlets may be the factors for such a high bacterial count. Butter and curd being produced by fermentation of milk may contain high number of fermenting microorganisms. However, butter may be contaminated with microorganisms coming from cream (Jay, 1996). In our study, four isolates including one from butter was gram negative in nature.

Reports are there of psychrotrophic gram negative bacteria developing and resulting proteolytic and lipolytic changes in butter (ICMSF, 2005). The microbiological examination of cooking butter revealed that 100, 36.7, 31.7, 31.7 and 23.3 % of the examined samples were contaminated by psychrotrophic bacteria, coliforms, faecal coliforms, *E.coli* and *S.aureus*, respectively (Meshref, 2010). Marketed fruit juice in Ogun state, South Western Nigeria have been found to be contaminated with *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Pseudomonas aeruginosa*, *Bacillus cereus*, *Enterobacter* sp., *Salmonella* sp., *Streptococcus* sp. and *Serratia* sp. (Bello et al., 2014). Contaminating pathogens in fruit juice may come from utensils like cutting board, knife and extractors (Martínez-Gonzales et al., 2003).

Lowest temperature for growth of all the twenty eight strains isolated lies between above 5°C to 15°C. Highest temperature for growth of two isolates from butter, three from curd, four from fruit juice and all the isolates from milk lies between 35°C to below 45°C. Four isolates from butter, six from curd and one from fruit juice had highest growth temperature of 45°C to below 55°C. Two isolates from butter had a highest growth temperature of 55°C. These characters indicate that heat treatment has killed the psychrophilic microorganisms may be present initially in the raw milk and the organisms present in the final product are either mesophilic or psychrotrophic. Presence of spoilage causing psychrotrophic bacteria in milk has been reported by Sørhaug and Stepaniak (1997).

All the eight isolates from butter could produce amylase at both 15°C and 25°C. One of them showed amylase production even at 35°C. While six of the isolates from curd produced amylase at both 15°C and 25°C, three at 15°C only. Of the five isolates from fruit juice, one produced amylase at 15°C only while two each at maximum of 25°C and 35°C, respectively. All the six isolates from milk produced amylase at both 15°C and 25°C. Two of them could produce amylase at 35°C also. Isolates BM5 from butter, FM4 and FM5 from fruit juice and MM1 and MM4 from milk produced amylase at all the temperatures ranging from 15°C to 35°C.

Proteinaceous compounds present in foods including simple proteins, conjugated proteins and large peptides are hydrolyzed to small peptides and amino acids by microbial extracellular proteinases and peptidases before cellular transport where they are converted to amino acids before further metabolism. Metabolic product of amino acids associated with spoilage include indole and skatole from tryptophan, putrescine and cadaverine from lysine and arginine, histamine from histidine, tyramine from tyrosine and sulphur-containing compounds from cysteine and methionine (Ray and Bhunia, 2007).

In this study, three of the isolates from butter while two from curd, produced caseinase at 35°C. One isolate from curd produced caseinase at 25°C. Only two of the isolates from milk produced caseinase at temperatures of both 25°C and 35°C.

Bacillus cereus, a gram positive and motile rod, commonly found in soil, has been frequently found in pasteurized milk, causing spoilage due to production of protease (Granum et al., 1993). None of the isolates from fruit juice could produce caseinase. No isolate could produce caseinase at 15°C. While six of the isolates from butter could produce lipase at 25°C, rest two could not produce lipase at all. One of the isolates from curd produced lipase at both 25°C and 35°C but all other isolates produced lipase at 25°C only.

None of the isolates from fruit juice and milk could produce lipase. No isolate could produce lipase at 15°C. Though lipase producing strains could not be isolated from fruit juice and milk isolates, Chang and Kang (2004) reported the presence of a lipase producing strain of *Alicyclobacillus acidoterrestris*, a thermoduric soil borne bacterium that produces off-flavour when growing in fruit juices.

The isolates showed resistance against a range of two to six numbers of antibiotics. While one (3.58%) of the isolates was resistant to six antibiotics, thirteen (46.4%) of the isolates including two from butter, four from curd, one from fruit juice and six from milk were resistant to five antibiotics. Eight (28.6%) of the isolates of which five were from butter, two from curd and one from fruit juice showed resistance to four antibiotics.

Four (14.3%) and two (7.1%) isolates were resistant to three and two antibiotics, respectively. All the milk isolates were resistant to five antibiotics. Munsch-Alatossava and Alatossava (2007) in their study found that out of 60 psychrotrophic bacteria isolated from raw milk, 60% harboured multidrug resistant traits. In a study on 32 staphylococci isolated from fruit juice samples, 78% were antibiotic resistant (Sharafati-chaleshtori et al., 2010). Considering the cell wall synthesis inhibitors, all the strains were sensitive to imipenem but resistance to ticarcillin and ampicillin were 93% and 100%, respectively.

Ninety-two percent of the isolates were resistant to cell membrane synthesis inhibitor antibiotics. This is supported by earlier study where 100% of the 48 foodborne *Bacillus cereus* isolates from legume-based fermented foods were resistant to polymyxin B (Roy et al., 2007). The tested isolates were mostly susceptible to antibiotics inhibiting protein synthesis and nucleic acid synthesis. Similar result was obtained by Tesfaw et al (2013), where *Salmonella* isolated from dairy products including cheese, butter and milk were sensitive to gentamicin and ciprofloxacin.

Finally it can be concluded that all the isolates had spoilage potential producing at least one of the three types of extracellular enzymes at a minimum of 15°C. Of these four of the isolates (14.3%) viz. BM5, BM8, CM1 and CM2 produced all the extracellular enzymes.

Antibiogram revealed that BM5 was resistant to four antibiotics, BM8 and CM2 against five antibiotics and CM1 was resistant to six antibiotics. Thus the refrigerated foods studied contain bacteria with spoilage potential and multiple drug resistance. So the foods should be stored below 15°C to avoid microbial spoilage.

Table.1 Growth of the isolates at combinations of different temperature and time

Source	Isolate	Temperature and time																	
		5°C			15°C			25°C			35°C			45°C			55°C		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Butter	BM1,BM3	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
	BM2	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-
	BM4, BM5	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+
	BM6, BM7, BM8	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Curd	CM1, CM3, CM4	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-
	CM2, CM5, CM7	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
	CM6, CM8, CM9	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Fruit juice	FM1, FM2, FM3, FM4	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
	FM5	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-
Pasteurized milk	MM1, MM2, MM3, MM4, MM5, MM6	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-

Table.2 Amylase production of the isolates at combinations of different temperature and time

Source	Isolate	Temperature and time								
		15°C			25°C			35°C		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Butter	BM1,BM2, BM3, BM4, BM6, BM7, BM8	-	+	+	-	+	+	-	-	-
	BM5	-	+	+	-	+	+	-	+	+
Curd	CM1, CM2, CM3, CM4, CM5, CM9	-	+	+	-	+	+	-	-	-
	CM6, CM7, CM8	-	+	+	-	-	-	-	-	-
Fruit juice	FM1, FM3	-	+	+	-	+	+	-	-	-
	FM2	-	+	+	-	-	-	-	-	-
	FM4, FM5	-	+	+	-	+	+	-	+	+
Pasteurized milk	MM1, MM4	-	+	+	-	+	+	-	+	+
	MM2, MM3, MM5, MM6	-	+	+	-	+	+	-	-	-

Table.3 Caseinase production of the isolates at combinations of different temperature and time

Source	Isolate	Temperature and time								
		15°C			25°C			35°C		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Butter	BM1, BM5, BM8	-	-	-	-	-	-	-	+	+
	BM2, BM3, BM4, BM6, BM7	-	-	-	-	-	-	-	-	-
Curd	CM1, CM2	-	-	-	-	-	-	-	+	+
	CM3, CM4, CM5, CM6, CM8, CM9	-	-	-	-	-	-	-	-	-
	CM7	-	-	-	-	+	+	-	-	-
Fruit juice	FM1, FM2, FM3, FM4 FM5	-	-	-	-	-	-	-	-	-
Pasteurized milk	MM1, MM3, MM4, MM6	-	-	-	-	-	-	-	-	-
	MM2, BMM5	-	-	-	-	+	+	-	+	+

Table.4 Lipase production of the isolates at combinations of different temperature and time

Source	Isolate	Temperature and time								
		15°C			25°C			35°C		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Butter	BM1, BM2	-	-	-	-	-	-	-	-	-
	BM3, BM4, BM5, BM6, BM7, BM8	-	-	-	-	+	+	-	-	-
Curd	CM1	-	-	-	-	+	+	-	+	+
	CM2, CM3, CM4, CM5, CM6, CM7, CM8, CM9	-	-	-	-	+	+	-	-	-
Fruit juice	FM1, FM2, FM3, FM4 FM5	-	-	-	-	-	-	-	-	-
Pasteurized milk	MM1, MM2, MM3, MM4, MM65, MM6	-	-	-	-	-	-	-	-	-

Table.5 Antibiogram of the isolates (n=28) from some refrigerated foods

Mechanism of action	Antibiotic (disc $^{-1}$)	No. of isolates		
		Sensitive	Intermediate	Resistant
Cell wall synthesis inhibitor	Ampicillin (10µg)	0	0	28
	Ticarcillin (75µg)	2	0	26
	Imipenem (10µg)	28	0	0
Cell membrane synthesis inhibitor	Polymyxin B (300U)	4	0	24
	Colistin (10µg)	4	0	24
Protein synthesis inhibitor	Streptomycin (30µg)	28	0	0
	Amikacin (30µg)	26	0	2
	Gentamicin (10µg)	27	0	1
	Nitillin (3 µg)	27	1	0
	Tetracycline (30µg)	5	10	13
Nucleic acid synthesis inhibitor	Ciprofloxacin (5µg)	26	1	1

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