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Isolation, Biochemical Characterization & 16s DNA Amplification of Different Bacterial Isolates from Milk and Milk Products

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ABSTRACT

Milk contains high nutritive value content such as protein, carbohydrates, and minerals. In addition to all these a protein called casein is also present in it which is only found in milk ad it is valuable for human health. The microbes present in milk responsible for the spoilage and they are also destroying the nutritive value by their enzymatic activity and metabolic reactions. So it was challenging issue to overcome these problems for microbiologist and scientists for prevent the growth and entry of these hazardous microbes in the milk and their product. Bacteria like heterofermentive lactobacilli, psychotropic bacteria, gram negative bacteria, yeast, molds, other microbes produce off odour in milk which cause sour taste and are unhygienic for human consumption and results in causing different disease like typhoid, diarrhea, brucellosis, tuberculosis. For identification of such life threatening bacteria microbiologist and science technologist investigate best way to find out the main reason for the spoilage milk and milk products prepared by them through several technical and experimental methods. These methods include bacterial isolation, identification, characterization, Biochemical test, morphological identification and 16s DNA amplification of isolated bacterial colonies from milk and their products. Study showed and proved that all bacteria are not harmful for human health some bacteria are also beneficial so to restrict or destroy harmful microbes several methods and experiments are performed during the processing of milk. These process of destroying harmful microbes include pasteurization, cooling, use of antimicrobial preservatives and chemical methods. These process doesn't make any change in milk taste and quality of milk. The main aim of this research study of these bacterial isolates for their microbial activity in milk to prevent health related issues in human and to overcome problems in their day to day life due to consumption of spoiled milk and milk products which are affected by these life threatening microbes.

Keywords: spoilage, psychotropic bacteria, Lactobacillus, DNA Amplification, casein.

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INTRODUCTION

Milk contains about 87% water and 13% solids. The fat portion of the milk contains fat soluble vitamins. The solids other than fat include proteins, carbohydrates, water soluble vitamins, and minerals. These nutrients in milk help make it nature's most nearly perfect food. Milk products contain high quality proteins. The whey proteins constitute about 18 percent of the protein content of milk. Casein, a protein found only in milk, contains all of the essential amino acids. It accounts for 82 percent of the total proteins in milk and is used as a standard for evaluating protein of other foods. The wide array of available dairy foods challenges the microbiologist, engineer, and technologist to find the best ways to prevent the entry of microorganisms, destroy those that do get in along with their enzymes, and prevent the growth and activities of those that escape processing treatments. The type of spoilage microorganisms differs widely among dairy foods because of the selective effects of practices followed in production, formulation, processing, packaging, storage, distribution, and handling. (Ledenbach and Marshall, 2009). The spoilage of pasteurized milk is caused by the growth of heat-resistant streptococci utilizing lactose to produce lactic acid, which depresses the pH to a point (about pH 4.5) where curdling takes place (Singh,Kaushal,et.al;2011)

Approximately 50% of the milk produced is consumed as fresh or boiled, one sixth as yoghurt or curd and remaining is utilized for manufacturing of indigenous varieties of milk products such as Ice cream, Butter, Khoa, Paneer, Rabri, Kheer, Burfi and Gulabjaman. Milk is such delicately flavoured, easily changed food that many preservative methods cannot be used without causing an undesirable change or at best making a different food product .After microorganisms have entered milk, it is difficult to remove them effectively. The process of centrifugation, as in clarifying or separating, will remove some microorganisms from milk. (Soomro,Arain, et.al; 2002). The first and most universal change effected in milk is its souring. So universal is this phenomenon that it is generally regarded as an inevitable change which cannot be avoided, the phenomenon is well understood (H.W.Conn). It is due to the action of certain of the milk bacteria upon the milk sugar which converts it into lactic acid, and this acid gives the sour taste and curdles the milk.

Natural souring of milk may be advantageous: for example, in smallholder butter-making, the acid developed assists in the extraction of fat during churning. The low pH retards growth of lipolytic and proteolytic bacteria and therefore protects the fat and protein in the milk (Frank O'Mahony 1988). Milk is one of the widely consumed nutrient food and also it is an excellent Culture medium for the growth and reproduction of microorganisms. Microbial growth can be controlled by cooling the milk. Most micro-organisms reproduce slowly in colder environments. Cooling milk also slows chemical deterioration. The

temperature of freshly drawn milk is about 38°C. Bacteria multiply very rapidly in warm milk and milk sours rapidly if held at these temperatures. If the milk is not cooled and is stored in the shade at an average air temperature of 16°C, the temperature of the milk will only have fallen to 28°C after 3 hours. Cooling the milk with running water will reduce the temperature to 16°C after 1 hour. At this temperature bacterial growth will be reduced and enzyme activity retarded. Thus, milk will preserved for longer period if cooled (C. O'Connor - 1995).

The significance of this study is to generate information about the microbial quality of selected milk and milk products in the town to indicate the current health risk. In addition, the study provides information for further study. It is important that researchers inquire and make recommendations to avert possible outbreak of food poisonings in retailed milk and milk products. We envisaged the need to examine causative agents of deterioration in milk and milk products. It was hypothesized that milk and milk product spoilage sets in as a result of microbial survival. Collection of milk and milk product samples from the local market area of Lucknow, Isolation and purification of bacteria from milk and milk products, Cultural Characterization of isolated strains of bacteria, Biochemical Characterization of isolated strains of bacteria, DNA Extraction from Pure Culture, Identification of bacteria isolated from milk and milk product samples. All these research are going to perform in this research study.

MATERIAL AND METHOD

All the materials, reagents and media for the study were procured from Himedia, Rankem, Thermofisher and SDFL, India.

Collection of Milk Sample and Product

A total three samples were collected from spoiled curd, Paneer and raw cow milk. All these three samples were spoiled curd is 3 weeks spoiled, paneer 9 days spoiled and raw milk is 1 day spoiled. Then all these samples were stored at room temperature. Samples were bought from street vendors of local market of Lucknow (U.P.). Each sample was placed separately in sterile plastic bags and immediately transported to the laboratory for processing within 1 h of collection. They were brought to the laboratory for microbiological analysis. Included in the analysis performed were isolation, biochemical characterization and screening.

Spoiled Sample for Analysis

All the samples selected were allowed to spoil for a definite period until they showed signs of spoilage.

Preparation of stock solution:

After suitable spoilage the stock solution was prepared either by sqeezing or crushing of each spoiled fruit and a homogenous suspension was obtained by thoroughly vortexing the mixture. The stock solution was prepared in sterile falcon tubes saperately.

Serial dilution:

The technique involved the removal of a small amount of an original solution to another container that is then brought up to a predetermined volume using the working solution (i.e. ddH_2O). To make a 1:100 dilution (10^{-2}), remove 10 µl and place this volume in a tube containing 990 µl of ddH_2O. This is often represented as 1:100 or 10-2.

To dilute this by a factor of 1:1000, remove 1 µl of the 1:100 dilutions and place it in a tube containing 999 µl of ddH_2O or media. The secondary concentration (1:100) has been diluted by a factor of 1,000 and the original solution has been diluted by a factor of 100,000 (the dilution factor). Same process was repeated to obtain 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions.

Media Preparation

Nutrient agar was the main medium used for the isolation of bacteria, some other important selective media were also used in the bacterial isolation process such as: Eosin-Methylene blue (EMB), Mac-Conkey agar, Mannitol Salt Agar, MRS

Nutrient Agar

It can be used for the cultivation and enumeration of bacteria which are not particularly fastidious.

Composition:

Peptic digest of animal tissue (5.000)gm/L, Sodium chloride (5.000)gm/L Beef extract(1.500)gm/L, Yeast extract (1.500)gm/L, Agar (15.000)gm/L, Final pH (at 25°C) 7.4±0.2.Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mixed well before pouring. (www.google.com)

Selective Media

Mannitol salt agar, EMB Media, De Man, Rogosa, and Sharpe (MRS) agar medium.

Isolation of microorganisms from spoiled sample:

The milk product samples were diluted quantitatively so that the total number of colonies on a plate ranged between 30 and 300. For few samples plates suitable for counting were obtained by planting 1ml and 0.1ml of undiluted sample and 1ml of sample diluted (ratio, 1:100). The cover of the sterile Petri dish was lifted just high enough to insert the piper. A sterile pipette was used to transfer measured diluted sample of milk products. The pipette was removed without retouching it to the plate; then 15 to20ml of the melted culture medium of between 44^{o}Cwas added. The plate was gently rotated for thorough distribution of inoculum

through the medium. The plate was incubated in inverted position in an incubator for 24H. The number of colonies counted on plate multiplied by dilution of sample gives the number of bacteria per ml.

The medium which was selected for the lactic acid bacteria was de Man, Rogosa, and Sharpe (MRS) agar medium. A loopful of the curd samples was streaked on the sterile MRS agar Petri plate by quadrant streaking method, under aseptic conditions. After streaking all the Petri plates, they were incubated at 37°C for 24 to 48 h. After the incubation, colonies were restreaked on the MRS agar Petri plate for the formation of isolated colonies. Then from these plates isolated colonies were restreaked on MRS agar slants and stored at 4°C.

Morphological characterization:

Microscopic examination By Gram staining

Cell morphology, cell arrangement, cell size, motility, presence of spores, and Presence of cell granules were determined by phase microscopy. Stained mounts were used for determining the Gram reaction and spore formation.

Biochemical Tests

Oxidase test, Catalase Test, Triple Sugar Iron Agar ,Sugar fermentation, Gas production, Hydrogen sulfide production ,Dimethyl Red and Voges Proskauer (MR-VP) **,**Citrate Test**.**

Concentration of DNA Samples:

Mainly DNA samples are concentrated by ethanol precipitation. In the presence of salt (mainly monovalent cations) and at low temperature, absolute ethanol precipitates nucleic acid. The acetate group replaces the hydroxyl group of water and DNA is able to make hydrogen bonds and becomes heavier. It can be easily separated by centrifugation or by spooling.

Gel Electrophoresis:

Agarose solution (50ml) was poured on a presealed glass plate. The gel was allowed to set and then comb were removed carefully. 1X TAE (Running) buffer was poured into chamber to cover the gel and fill the wells. Prepare DNA sample by adding 8µl loading dye (bromophenol blue) and 5µl DNA and mixed well and then load the prepared DNA sample in wells. Connected the electrode to power plug at constant voltage of 50V for 2-3 hrs and allowed the sample to move under the influence of electric field. The mobility of sample was tracked by movement of bromophenolblue and electrophoresis was allowed to continue till the tracking dye reached 3/4th of the gel length and finally examined the gel under gel doc.

Identification of bacterial isolates by 16S rDNA amplification and sequencing of the amplified products:

The DNA isolated, as above, was amplified with the PCR System (Bio-Rad, USA) using 16s rDNA universal primers and sequenced for the identification of bacterial strain at molecular level. The 16S rRNA genes were amplified by polymerase chain reaction The PCR thermal cycling programme used was as follows: .initial denaturation at 95^{o}C for 5min; 30 cycles of denaturation, annealing and extension at 94^{o}C, 52^{o}C and 72^{o}C for 30s, 30s and 1min 25s respectively, followed by a final extension at 72^{o}C for 10min and kept at a hold temperature of 4^{o}C. All the amplifications were carried out in triplicates. Amplification of the PCR products of expected size was confirmed by electrophoresis. The sequence of the 16S rDNA was determined by Amnion Biotech Inc, Bangalore, India. The gene sequences of each isolate obtained in this study were compared with known 16s rRNA gene sequences in the GenBank database.

RESULTS AND DISCUSSION

The present study was aimed to isolate and characterize different bacterial isolates from different milk and milk products.

CFU Count:

The number of lipolytic organisms per gram was estimated by standard Formula Dilution factor (1: 10):

No. of microorganisms / gram = No. of Colonies × Dilution Factor/ Volume of sample added

The no. of CFU was found to be in spoiled curd, Paneer and cow milk sample.

Table 1: Showing CFU count of bacteria isolated from spoiled samples

Sample	Isolate code	No. of colonies	Dilution Factor	CFU count
Curd	A1	72	10^{-5}	$7.2x10^{-7}$ Cfu/ml
Paneer	B1	5	10^{-6}	$0.5x10^{-8}$ Cfu/ml
(a)Cow milk 10-6	C1	83	10^{-6}	
(b) Cow milk 10-7	C2	64	10^{-7}	

Colony morphology:

Furthermore, when spoiled milk and milk product sample were spreaded on NA plate we were observed different texture, size, shape and colour colonies. Some colonies were round, opaque, elevated, creamy colonies of microorganisms.

Sample 1: Curd

Figure 1 :(a) showing spoiled curd sample of 10^{-5}& 10^{-6} dilutions spreaded on nutrient agar media

(b)Paneer sample 10-7 dilution NA plate(c) Cow milk 10-7 dilution NA Plate

Table2: Texture and Morphological Characteristics of Bacterial Isolates:

Sample	Isolate code	Medium	Elevation	Edge	Size	Colony color
Curd	A1	NA	Raised	Irregular	small	White
Paneer	B1	NA	Flat	Regular	large	White
Cow milk	C1	NA	Raised	Entire	small	White

De Man, Rogosa, and Sharpe (MRS) agar medium

We further wanted to find out the presence of some lactobacillus species in our sample (curd, Paneer and cow milk). So, we further spreading of serially diluted samples on MRS agar Plate for lactobacilli. Colonies of lactic acid bacteria were observed on the surface of MRS plates. More than one colony was observed in most of the cases. Cultural and morphological characteristics wereexamined with the help of microscope. Different types of microorganisms were observed, majority of them belonged to Gram+ve rods and *cocci* shaped bacteria. The purification of isolates was done by transferring Gram+ve rods and *cocci* shaped bacteria to the plates of selective media MRS respectively. These isolates were further sub cultured until pure isolates were obtained.

From the tested samples, lactic acid producing strains were isolated from different milk product samples. Colonies were observed on the surface of MRS agar Petri plate. More than

one type of colony was observed on surface of MRS agar Petri plate. Small and large two types of colonies were observed. Most of colonies were creamy to white. The cultural and morphological characteristics were further resolved on the basis of microscopic examination. Majority of the microorganisms were Gram positive rods and cocci.

On observing selectivity results of different milk product samples grown on selective media it was observed that curd and paneer sample gives negative result on mannitol salt agar and EMB media whereas cow milk gives positive results on both media.

Identification test of *bacteria*

All isolates were microscopically examined for gram stain reaction, cell morphology and cellular arrangement.

GRAM'S STAINING:**Sample-1(curd)** :

Rod shaped cells was observed with pink colour. Therefore it was identified as Gram negative bacteria.

Sample-2 (Paneer) :

Rod shaped cells was Observed with pink colour. Therefore it was also identified As Gram negative bacteria.

Sample-3 (cow milk):

Rod shaped cells was Observed with purple colour. Therefore it was also identified As Gram positive bacteria.

BIOCHEMICAL TESTS

Triple sugar iron test:

The triple sugar- iron agar test is designed to differentiate among the different groups or genera of the Enterobacteriaceae, which are all gram negative bacilli capable of fermenting glucose with the production of acid, and to distinguish them from other gram negative intestinal bacilli.

Table-3: TSI Test Result

Sample	Isolate Code	Slant	Butt	Hydrogen sulphide	Suspected species
Paneer	B1	Y	Y	-	*S.aureus*
Curd	A1	R	YG	+	*Salmonella*
Cow Milk 10^{-6} dilution	C1	Y	YG	-	*Ecoli, Klebsiella Pneumoniae*
Cow Milk 10^{-7} dilution	C2	Y	YG	-	*Ecoli, Klebsiella Pneumoniae*

Table 4: showing biochemical characterization of different bacterial isolates from spoiled Milk and milk product samples:

Kind of sample	Isolate code	Catalase test	Oxidase test	Methyl Red test	Voges Proskauer test	Indole test	Simmons citrate test
Curd	A1	+	-	+	-	+	+

Paneer	B1	-	-	+	-	-	-
Cow milk	C1	-	+	+	+	+	+
	C2	-	+	+	+	-	+

Sugar fermentation test:

Carbohydrate fermentation tests detect the ability of microorganisms to ferment a specific carbohydrate.

It was observed that paneer sample(B1) shows negative result for glucose fermentation and positive results for lactose and mannitol fermentation similarly on observing curd sample(A1) and cow milk sample (C1 and C2) gives positive results for all these sugar fermentation test.

Bacterial Typing by 16S rDNA Amplification:

In order to identify bacterial isolates at the species level we further aimed to analyse the 16s ribosomal DNA amplification for bacterial typing. In this scenario, we first of all isolated the bacterial genomic DNA from different isolates by a following generalized protocol for the isolation of bacterial genomic DNA. The isolated bacterial DNA was visualized on agarose gel containing stained with ethidium bromide (Etbr) as shown in figure15. Bands of bacterial genomic DNA were observed on agarose gel containing ethidium bromide (EtBr).

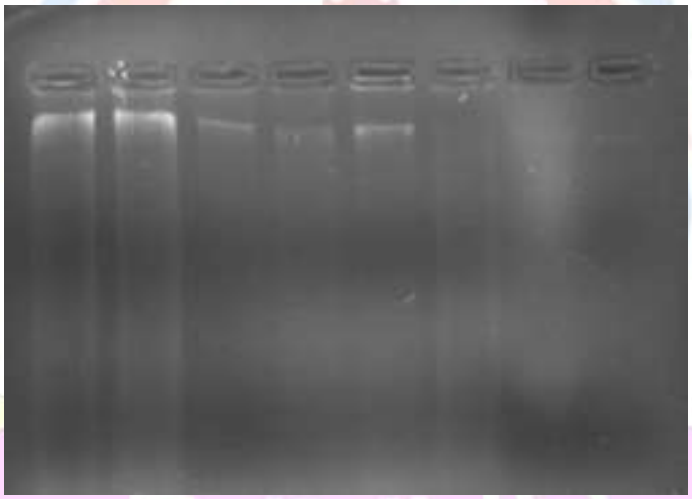

Figure 2 : showing genomic DNA bands on agarose gel.

Quantification:

The amount of genomic DNA was quantified according to the formula as earlier discussed in material and methods and the purity of DNA was checked and was found to be between the ranges:

Medium	Isolates	Isolate Code	A_{260}	A_{280}	260/280 Ratio λ1/λ2	DNA Concentration µg/ml
NA	Paneer	B1	0.214	0.161	1.329	107
	Cow milk	C1	0.045	0.016	2.81	22.5
	Curd	A1	0.514	0.289	1.77	257
	Paneer small	B2	0.310	0.167	1.85	155

MRS	Cow milk	C1	0.347	0.174	1.9	8.5
	Curd	A1	0.881	0.605	1.45	440.5
	Paneer	B1	0.913	0.551	1.65	456.5
EMB	EMB Cow	C2	0.314	0.194	1.75	170.5

(DNA Concentration= 50µg/ml × A_{260}× dilution factor)

CONCLUSION:

From this research study we conclude that Bacteria are contaminants of milk and milk products. In order to avoid excessive spoilage, various measures can be employed to kill bacteria or to retard bacterial growth. These include keeping foods cold (or frozen), boiling (as is done for canned foods), salting (pickling), dehydrating (as in beef jerky), and adding anti-bacterial preservatives. In the particular case of milk, pasteurization combined with refrigeration is the most common technique used. Pasteurization does not kill all the bacteria (or spores) in milk, but does eliminate most of the pathogenic bacteria that have been historically associated with milk, such as tuberculosis, brucellosis, and typhoid. Pasteurization was first developed in order to kill these pathogens, but it was soon discovered that this process also improved the keeping quality of the milk without sacrificing the taste.

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