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Study On Proteolytic Enzyme From Probiotic Non Lactic Acid Bacteria Isolated From Sheep Milk

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***Abstract*—Probiotics are live micro-organisms that "when administrated in adequate amount confer a health benefit on the host (WHO). The milk samples from sheep, buffalo, cow and goat were collected aseptically and 25 isolates were isolated in Tryptone soya agar. Among that, 10 isolates were confirmed for the probiotic proteolytic non lactic acid bacteria proteolytic zone in caseinate TSA medium. According to biochemical tests, identification and characterization of enzymes indicates that isolates might belong to *Enterobacteriaceae* and *Proteus* family. The test yielded 5 potent bacterial isolates from sheep milk which showed constant and maximum production in 1% caseinate TSA media supplemented with varied concentrations of MnCl, NaCl and ZnCl salts at 35°C. The Rf values of TLC profiling of proteolytic enzyme were obtained in the range from 0.81 to 0.87. The autobiography profiling of the TLC plates resulted in the formation of zone of inhibition confirmed for the proteolytic activity.**

***IndexTerms*—Autobiography, *Enterobacteriaceae*, Probiotics, *Proteus*, Proteolytic activity and TLC profiling.**

I. INTRODUCTION

The word 'probiotic' comes from Greek language 'Probios' which means 'for life' opposed to 'antibiotics' which means 'against life'. The history of probiotics began with the history of man by consuming fermented foods that is well known Greek and Romans consume very much. Probiotic is a live microbial supplement which affects host's health positively by improving its intestinal microbial balance. Than this definition was boarded by Havenaar and Huis that held in 1992 including mono or mixed culture of live microorganisms which applied for animal and man [1]. In October 2001, reported that probiotics are 'live microorganisms which when administered in adequate amount confer health benefits on the host', is accepted by FAO/ WHO [2]. The range of food products containing probiotic strains is wide and still growing. The main products existing in the market are diary based on including fermented milks, cheese, ice-cream, buttermilk, milk powder and yoghurt etc. Probiotics were showed reliable probiotics properties were examined for further probiotics properties including tolerance to different concentration of bile salts, NaCl, antimicrobial activity and bile salt hydrolase activity [3][4].

The defense mechanisms of microbes are of different types based on the nutritional and environmental parameters in which they reside. A number of factors mitigate the severity and survival rate of competition between the competitors. Microbial antagonism is defined as the suppression or interference of the normal growth of one microbe by another. The mechanism of antagonistic activity involves antibiosis, direct parasitism, competition and induced resistance.

Induced resistance is a mechanism where one microbiome gets mutualistic with the other of same or different species wherein they involve in the defense system against a broad range of microorganisms by associating with a selected host. The induced systemic resistance enhances the host immune system against the pathogens [5].

The defense mechanism of the microbial population mainly depends on the bioactive peptides released during the course of reproduction and growth. Bioactive peptides (BP) are defined as the protein fragment or a protein conjugate that have a positive impact on body's function and health condition by affecting the digestive, endocrine, cardiovascular, immune and nervous systems. They are the organic substances formed by covalently bond amino acids which are known as amide or peptide bonds [6]. Although few bioactive peptides exist free in its natural or native form, while the vast majority of them is encrypted in the structure of the parent proteins and is released mainly by enzymatic processes. The growing interest in bioactive peptides has incentivized the scientific community and nutraceutical to explore the development of new functional products based on these peptides [7].

Milk contains two proteinase system plasmin and lysosomal proteinase derived from bloods which are involved in dissolving blood clots and defense mechanism respectively. Proteases are group of enzymes which hydrolyses peptide bond of proteins and break them down into polypeptide or free amino acids. Proteases are present in all forms of life viz. plant, animals and microorganisms and are essential for cell growth and differentiation. Proteases are classified into exopeptidases and endopeptidases based on their ability to cleave peptide bonds. Exopeptidases cleave peptide bonds at C- and N- terminus and are further divided into aminopeptidases, dipeptidases, dipeptidylpeptidases,

tripeptidylpeptidases, peptidyldipeptidases and carboxypeptidases. Endopeptidaseshydrolyse internal, alpha-peptide bond in a polypeptide chain e.g. chymotrypsin, pepsin and paparin[8].

Most microorganisms recognized to date as probiotic are gram positive with *Lactobacillus* and *Bifidobacterium* being the main species used as treatment of intestinal dysfunction. However, some gram negative is also used as probiotics. The best example of this group is *E.coli*Nissle 1917 (EcN), *Pseudomonas aeruginosa* PIC4, *Streptococcus salivarius, Bacillus spp.* Sheep milk has high nutritional value and high concentration of proteins, fats, minerals and vitamins as compared to the milk of other domestic species. The physicochemical and nutritional characteristics of sheep milk can be advantages for the manufacture of products containing prebiotic ingredients and/or probiotic bacteria, which are major categories in the functional food market [9].

Enzymatic hydrolysis of milk proteins can release fragments that are able to exert specific biological activities such as antihypertensive, antimicrobial, opioid, antioxidant, immuno-modulatory or mineral binding. Such protein fragments, known as bioactive peptides are formed from the precursor inactive protein during gastrointestinal digestion and/or during food processing [10].

Balthazar *et al.,* (2017) has analyzed that agglutinin is absent in sheep milk and goat milk, providing better digestibility compared to cow milk. Sheep milk and goat milk have high concentration of fat globules, which are smaller than cow milk; these globules diameters average are approximately 3.6 and 3.0µm against 4.0µm, respectively. The size and dispersion of the fat globules confer greater consistency to this milk, favoring freezing without phase separation. Sheep milk is rich in casein (4.2 to 5.2g/ 100g) and whey proteins (1.02 to 1.3g/ 100g).

II. MATERIALS AND METHODS

Milk samples (cow, sheep, buffalo, and goat) was collected from village area and used as a raw material for isolation of proteolytic non-lactic acid bacteria.

Bacteriological Media

All microbiological media were procured from Hi-Media. Tryptone Soya Broth (Soyabean Casein Digest Medium), Nutrient broth and agar were the media used in the study. All the chemicals were of analytical grade.

Isolation and identification of proteolytic non-lactic acid bacteria from raw milk samples

Raw milk samples of 1 mL from cow, sheep, buffalo and goat were inoculated in nutrient broth and TSB (Tryptone Soya Broth) incubated in shaker incubator at 37 °C, 120 rpm for 24 hours [11]. The cultures were inoculated for isolating the proteolytic strains by identifying the bacterial colonies that gave clear zone on 1 % casein TSA medium. The isolated colonies were later subjected to biochemical and 16s RNA sequence identification [12].

Probiotic properties analysis [3]

The probiotic properties of the isolated bacteria were checked forNaCl tolerance, Bile tolerance, Phenol tolerance, Milk coagulation test and Antibiotic resistance test by disc diffusion assays.

Proteolytic activity at varied parameters

The most important aspects of secondary metabolite study are production and purification. Very often complex media compositions are used for the production of proteolytic enzyme that interferes in the purification. So there is a necessity for the optimization of multiple factors like biotic and abiotic factors for the high production of proteolytic enzyme, and is usually strain specific. The optimization process was carried out based on One-factor-at-a-time (OFAT) for both cultural and nutritional parameters [13].

- **Growth at different temperature**
- **Growth at different salts**
- **Growth at different concentration of casein**
- **Growth in different time intervals**

Solvent extraction of the cell free supernatants: Semi-polar solvents like acetone and butanol were mixed with cell free supernatant (1:1 v/v) separately based on the miscibility property [14].

Confirmation of protease by thin layer chromatography: The prepared crude enzymes were used for TLC. The protease production of isolates was confirmed by subjecting the solvents extract to thin layer chromatography (TLC). The separation and identification were carried out by using thin layer chromatography technique by using silica gel. The solvent system used for mobile phase was chloroform, methanol and water. Rf values were calculated [15].

TLC bioautography of various extracts: For the further confirmation of proteolytic activity of separated extract from TLC sheet. The TLC sheets were subjected to bioautography on casein agar medium for the clear zone around the TLC plate [16].

III. RESULTS AND DISCUSSIONS

Isolation and identification of proteolytic non-lactic acid bacteria from raw milk samples

The colonies appearing on soyabean casein digest medium with 1% of casein for isolation of non-lactic acid proteolytic bacteria. The Figure.1 shows colonies showing clear zone were sub cultured on soyabean casein digest agar for confirmation of proteolytic activity which showed clear zone.The purified proteolytic bacteria were streaked on tryptone soya agar slants in test tubes and kept at 4 °C for further tests. The proteolytic bacteria hydrolyzed the casein present in the media to produce the clear zone around the colonies indicating the proteolysis.

Probiotic properties analysis

The Graph.1shows thatisolates were screened for their capacity to tolerate different concentration of NaCl, bile salt

and phenol tolerance assays. The isolates were able to fairly grow at 1 -5% of NaCl whereas in 10% the growth drastically decreased. Isolates for bile salts analysis showed well growth at 0.05% to 0.3%. Isolates are defiant to phenol at different concentrations. At 0.1% all isolates showed higher level of tolerance, whereas in 0.2-0.3 % they were moderate but in 0.4% tolerance was much lower.

Proteolytic activity at varied parameters

The Graph.2 showszone of inhibition observed in tryptone soya agar supplemented with 1% casein at different temperature and significantly at 35 ° C. The growths of the two isolates in different salts at 35 ° C with increased zone of inhibition were observed in the media containing NaCl and MnCl salts.

The Graph.3 showed the 0.5- 1% casein concentration having high proteolytic activity with increased zone of inhibition and the increased activity was observed till 48th hours of incubation time.

Confirmation of protease by thin layer chromatography

The separation of proteins was based on the differential partition coefficient of stationary and mobile phase. The developed chromatogram was air dried and observed for the bands under ultra violet (UV) light at both short wave (254 nm) and long wave (366 nm). The movement of analyte was expressed as partition coefficient, indicated by retention factor (R_f). Figure.2 & Figure.3 shows the conformational studies by using thin layer chromatography reported that isolates with cell free suspension by using two solvents having Rf values were in the range of 0.81 to 0.87.

TLC bioautography of various extracts

The autobiographic analysis clearly indicated the partially purified proteolytic enzyme activity which was observed in Figure 3 by the formation of zone of inhibition in the casein media.

IV. CONCLUSION

Probiotics are live micro-organisms that "when administrated in adequate amount confer a health benefit on the host (WHO). Milk contain two proteinase system both derived from blood, one involved in dissolving blood clots (plasmin) and the other in defense against invasive micro-organisms (lysosomal proteinases of somatic cells). Both systems hydrolyze the caseins. Milk contains both lactic acid and non lactic acid bacteria which is showing some beneficial effects to the host as probiotics or prebiotics. The probiotic property of non lactic acid bacteria conducted showed a maximum growth curve indicating the tolerance and proved their survivality for different concentrations of bile salts, sodium chloride and phenol. The studies conducted by Radhika Pilli *et al.,* 2016 on the alkaline protease production and confirmation from fungi was confirmed by thin layer chromatography (TLC) of the extract and the Rf values in the range of 0.84 – 0.87. The work carried out was correlated with the above work which confirmed that the probiotic non-lactic acid bacteria produced the proteolytic enzyme which hydrolyzed the casein was active at alkaline pH which can be classified to be an alkaline protease.

FIGURES AND GRAPHS:

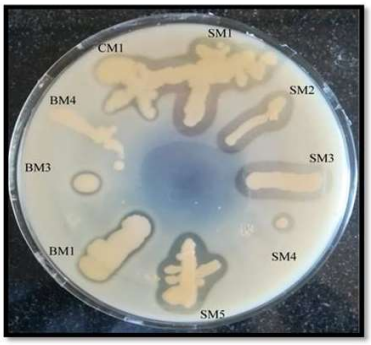
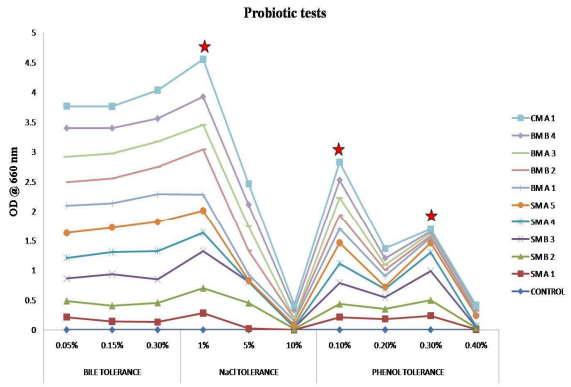
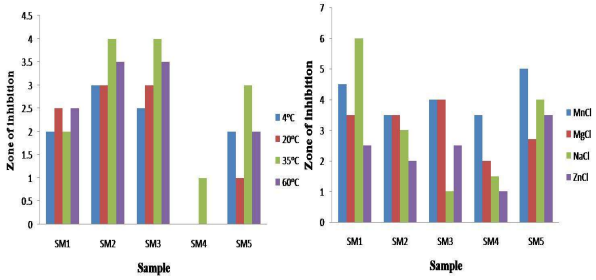


Figure 1: ISOLATION WITH 1% CASEIN IN TSA MEDIA

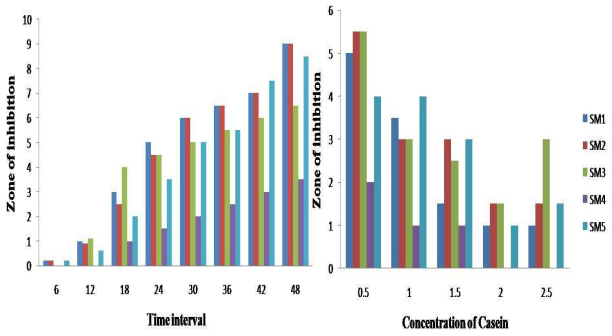


★ Indicates the highest growth

GRAPH 1: PROBIOTIC CONFIRMATORY ASSAY FOR THE PROTEOLYTIC ACTIVE BACTERIAL ISOLATES



GRAPH 2: PROTEOLYTIC ACTIVITY AT DIFFERENT TEMPERATURES AND VARIED SALT CONCENTRATION



GRAPH 3: PROTEOLYTIC ACTIVITY AT DIFFERENT TIME INTERVLAS AND CASEIN CONCENTRATION

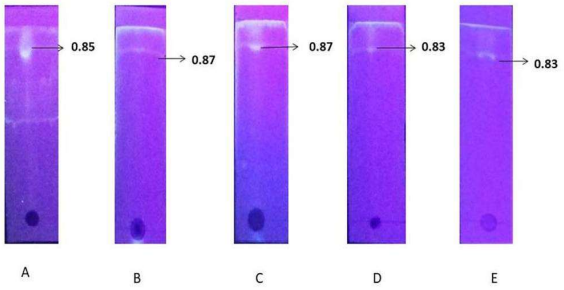


FIGURE 2: Rf VALUE OF THE EXTRACTS FROM ACETONE

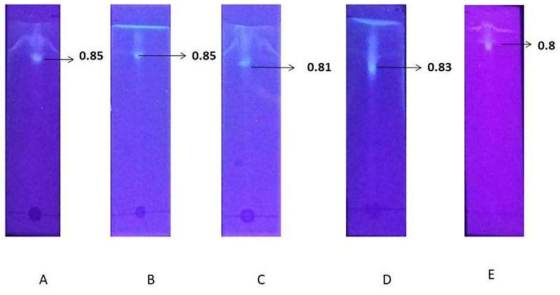


FIGURE 3: Rf VALUE OF THE EXTRACTS FROM BUTANOL

(A) SM1; (B) SM2; (C) SM3; (D) SM4 and (E) SM5

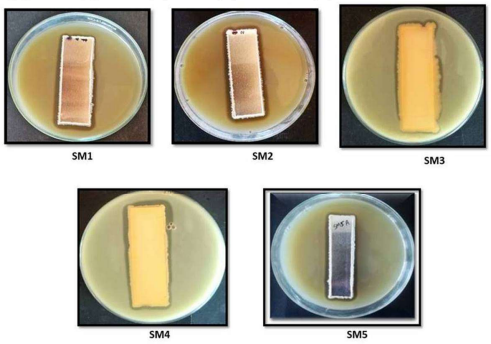


FIGURE 4: BIOAUTOGRAPHY OF VARIOUS EXTRACTS IN TRYPTONE SOYA AGAR SUPPLEMENTED WITH 1% CASEIN.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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