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Screening and Identification of Industrial Enzyme Producing Bacteria from Sheep and Goat Milk

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ABSTRACT: Industrial enzyme producing bacteria are essential for advancements in biotechnology. This study aims to identify bacterial strains from sheep and goat milk capable of producing industrially valuable enzymes. Basic Gram's Staining and motility tests were performed from the bacterial colony of samples. Milk samples yielded a colony of bacteria which were screened for protease and amylase production. IMVIC tests demonstrated the feasibility of utilizing sheep and goat milk as a rich source of industrially valuable enzyme producing bacteria. By using SDS – PAGE the presence of the protein molecules is found. This study contributes to the discovery of novel enzyme-producing bacteria from unconventional sources, providing opportunities for the development of sustainable and efficient industrial processes. In this case, the results suggest that sheep and goat milk can serve as a rich source of industrially relevant enzyme-producing bacteria, offering potential applications in food processing, textile, and pharmaceutical industries. This demonstrated that certain bacterial strains exhibited high enzyme activity, highlighting their potential for industrial applications. Further studies on optimizing enzyme production and understanding the genetics of these isolates could lead to the development of new microbial strains for efficient enzyme production in various industries.

Keywords: Industrial Enzyme, Goat Milk, Sheep Milk, IMVIC, Enzyme assays, Dairy Microbiology

I. INTRODUCTION:

Industrial enzymes play a crucial role in various sectors, including food, pharmaceuticals, textiles, and biofuels, accounting for approximately 50% of global enzyme production. The increasing demand for these enzymes has necessitated the exploration of novel, sustainable, and cost-effective sources of enzyme-producing microorganisms. Recent attention has shifted towards exploiting the untapped potential of livestock-associated microbiota, particularly those present in milk.

Sheep and goat milk, rich in nutrients and bioactive compounds, harbor diverse microbial communities that remain largely unexplored. These milk-derived microorganisms possess unique enzymatic capabilities, making them attractive candidates for industrial enzyme production. The isolation and characterization of enzyme-producing bacteria from sheep and goat milk can provide valuable insights into their potential applications in various industries.

Sheep and goat milk are valuable sources for industrial applications due to their unique composition and properties. Sheep and goat milk enzymes have garnered significant attention in recent years due to their unique properties and potential industrial applications. These enzymes, produced by microorganisms present in sheep and goat milk, offer a sustainable and innovative solution for various industries. With higher protein, calcium, and phosphorus content than cow milk, they support the human life in various ways. Sheep and goats have evolved to produce milk with unique enzymatic profiles to support their offspring's growth and development. Grazing on diverse plant species exposes sheep and goats to a wider range of enzymes, which are then transferred to their milk. Sheep and goats have shorter lactation cycles, resulting in more frequent milk production and higher enzyme concentrations.

Milk enzymes exhibit distinct industrial characteristics, making them valuable in various applications. These enzymes, including proteases, lipases, lactoperoxidases, and phosphatases, demonstrate temperature stability, pH tolerance, and substrate specificity. They optimize dairy processing, cheese production, and food

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manufacturing, improving product quality and efficiency. With benefits like reduced energy consumption and enhanced nutritional value, milk enzymes are increasingly used in pharmaceuticals, biotechnology, textiles, and cosmetics. Advances in recombinant technology, enzyme immobilization, and nanotechnology enhance their potential. However, scalability, standardization, and regulatory frameworks remain challenges. Industrial applications of milk enzymes are expanding, driven by advances in enzyme technology and growing demand for sustainable, efficient processes. In pharmaceuticals, milk enzymes aid drug development and vaccine production. Biotechnology applications include biocatalysis and biofuel production. Textile manufacturers utilize milk enzymes for wool and silk processing, while cosmetics and skincare industries exploit their anti-aging and skin repair potential.

Sheep and goat milk enzymes are preferred for industrial applications due to their unique characteristics, including higher enzyme activity, temperature and pH stability, and substrate specificity. These enzymes offer nutritional benefits, improved product texture and flavor, and enhanced processing efficiency. Their smaller molecular size and lower allergenicity make them suitable for sensitive applications. Additionally, sheep and goat milk enzymes support sustainable, local dairy practices. Key enzymes like lipase, protease, lactoperoxidase, chymosin, and phosphatase are utilized in cheese production, pharmaceuticals, cosmetics, textiles, and food processing. Their distinct fatty acid profile and easier absorption properties further enhance product value. Overall, sheep and goat milk enzymes provide a valuable combination of efficiency, sustainability, and product quality, driving their adoption in various industries.

II. METHODOLOGY:

Materials and Sample Collection

Sheep Milk

Fresh milk is collected from Nilgiris Sheep (Ovis aries nilagirica).

Nilgiris Sheep (Ovis ariesnilagirica) Milk

Goat Milk

Fresh milk is collected from Nilgiris Goat (Capra aegagrus hirucs nilagirica)

Nilgiris Goat (Capra aegagrus hirucsnilagirica) Milk

Nutritional Plates Techniques

Milk Agar Plate

A milk agar plate is a type of nutritional culture medium used to detect the presence of certain enzymes produced by microorganisms. This plate contains milk protein, casein, and is used to indicate the presence of the enzyme like Protease (Breaks down casein).

Milk Agar Plate

Potato Dextrose Agar Plate

Potato Dextrose Agar (PDA) is a solid, nutrient-rich medium composed of potato extract, dextrose (glucose), and agar, designed to support the growth of fungi and inhibit bacterial growth. A potato dextrose agar plate is used to detect the presence of enzymes like Amylase. This plate contains Potato extract, Dextrose, Agar. The enzyme breaks down the starch into sugars.

Potato Dextrose Agar

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Enumeration of Microorganisms

Preparation of Sample

The 100 µl of the test sample (Sheep milk, Goat milk) was taken using the spread plate method. The 10^4 and dilution was used for bacterial isolation.

Nutritional Agar Medium

The medium was prepared by dissolving Peptone- 1g, NaCl- 1g, Yeast extract- 0.5gm, Beef extract-0.2g, and agar powder- 1.75 gm of the commercially available Nutrient Agar Medium in 100ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm Petri plates (25-30ml/plate) while still molten. The 10^4 dilution was plated on the Nutrient agar medium by spread plate method and the plate was incubated at 37° C for 24 hrs. After incubation, bacterial colonies were isolated and plated into a fresh plate.

Colony Forming Unit (CFU)

The given sample (Goat milk and Sheep milk-100 µl) dilution 10^4 was taken using the spread plate method of test samples by spread plate method using Nutrient Agarcultured overnight at 37 °C in a bacteriological incubator.

$$\text{CFU/ml} = \frac{(\text{No. of colonies} \times \text{Total dilution factor})}{\text{The volume of culture plated in ml}}$$

Gram's Staining

A loop full of bacterial culture was spread in the glass slide. The slide was smeared in front of the flame. The slides were stained with crystal violet dye kept it for 1 min and washed the slide in distilled water. Gram's iodine was added and incubated for 1 minute, then rinsed with distilled water. The decolorizing agent was added and kept for 1 min and then safranin strain was added, after a minute it was washed using distilled water. The slides were observed under the Trinocular microscope the purple colors indicated gram-positive bacteria and the pink color indicated gram negative organisms

Crystal Violet

⬇

Grams Iodine

⬇

Ethanol

⬇

Safranin

IMVIC Tests (Physio Chemical Characterization)

Indol Test

The Indol test is performed to detect the formation of tryptophan by the enzymatic reaction of tryptophanase.

Tryptophan + Distilled Water = Indol +Pyruvic acid + Ammonia

Procedure

Inoculate the bacterium to be tested in tryptone broth. Incubate at 55°C for 24 hrs after incubation add a few drop of Kovac's reagent. The formation of a red colour ring (or) pink colour ring at the top denotes a positive reaction and yellow colour ring at the top denotes negative result.

Methyl Red Test

Methyl red test is to check the ability of bacteria to perform mixed acid fermentation of glucose. It can check the fermentation by indicating the pH.

Procedure

Inoculate the bacterium to be tested in MRVP broth. Incubate at 55°C for 24hrs after incubation add 5 drops of the methyl red reagent. The formation of the red colour ring on top indicates a positive reaction, formation of yellow colour ring at the top indicates negative reaction.

Voges Proskauer Test

The VP test is bacteriological test used to detect the presence of the enzyme butyraldehyde dehydrogenase, which is produced by certain bacteria. It detects the production of acetoin from butyraldehyde, which is then converted to diacetyl.

Procedure

Inoculate the bacterium to be tested in MRVP broth. Incubate at 55°C for 48hrs. After incubation add 0.6ml of alpha naphthol, and 0.2ml of 40% KOH to the broth. Allow tube to stand for 15 minutes. Formation of red colour ring shows

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positive result and the formation of a yellow color ring shows a negative reaction.

Simmon's Citrate Utilization test

The citrate test is a bacteriological test used to determine the ability of microorganism to utilize citrate as a sole source of carbon.

Procedure

Bacterial colonies were inoculated into a slant of Simmon's Citrate agar using an inoculation loop. Incubate at 55^{o}C for 24 hrs. Finally, if the medium colour changes from green to blue, it implies positive reaction; if colour changes from green to yellow, it implies a negative reaction.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is used to analyze protein mixtures qualitatively. It is particularly used for monitoring the purity of protein and to determine the relative molecular mass of proteins. Samples to be run on SDS-PAGE initially are treated for 3 min at 90 C in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduces the disulfide bonds present in the tertiary structure of proteins. SDS (CH_3-$(CH_2)_{10}$-$CH_2OSO_3^-$ Na^+) is an anionic detergent, which binds strongly to and denatures the protein. It has been found that 1.4g of SDS binds 1g of protein and renders a net negative charge. The sample buffer also contains an ionizable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored, and sucrose or glycerol aids the protein sample to settle on the bottom of the loading well by increasing its density. Once the samples are loaded current is applied through a gel.

The samples to be separated are loaded in stacking gel, which is about 1cm long to stack the proteins in the sample into a sharp band before it enters the resolving gel, which is about 10cm long. Stacking is achieved by utilizing differences in ionic strength and pH between the electrophoresis buffer and the stacking gel and involves a phenomenon known as isotachophoresis. The stacking gel has a very large pore size (5% acrylamide), which allows the protein to move freely and concentrate, stacking under the effect of the electric field. The band sharpening effect relies on the fact that negatively charged glycinate ions (in the electrophoresis buffer) have lower electrophoretic mobility than the protein-SDS complexes, which in turn have lower mobility than the chloride ions of the loading buffer and the stacking gel.

When the electric field is applied, all the anions migrate towards the positive end (bottom of the gel). The glycinate ions can only move at the same speed as chloride ions if they are in a region of higher field strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. There is only a small quantity of protein-SDS complexes, so they concentrate in a very tight band between glycinate and chloride boundaries. Once the glycinate reaches the separating gel it is neutralized and stops its movement. The negatively charged protein SDS complexes now continue to move a bit slowly compared to stacking gel towards the anode, and because they have the same charge per unit length they travel into the separating gel under the applied electric field with different mobility owing to the difference in their molecular weight. Smaller proteins can easily pass through the pores of the gel, whereas large proteins are retarded by the frictional resistance of the gel. Being a small molecule the bromophenol blue dye can move freely without any friction. Thus it marks the fore front of the run.

Structure of SDS- PAGE apparatus

Materials Required for SDS-PAGE

Separating gel buffer:

1.5 M Tris base - 18.15 g

Adjusted the pH to 8.8 using HCl and made up to 100ml

Stacking gel buffer:

0.5 M Tris base - 6 g

Adjusted the pH to 6.8 using HCl and made up to 100ml

Electrode buffer (10X):

Tris base	30.3 g
Glycine	144 g
SDS	10 g

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Made up to 1000 ml with distilled water. The pH was adjusted to 8.3 with 10 N NaOH. The buffer was diluted to 1X with distilled water, whenever required.

Acrylamide (30%):
Acrylamide - 29.2 g
Bis acrylamide - 0.8 g
Make up to 100 ml using distilled water

APS (10%):
100 mg in 1 ml water

SDS (10%):
10 g in 100 ml distilled water.

Sample buffer (2X):
100 mM Tris-HCL - 0.121 g
4% SDS - 0.4 g
0.2 % Bromophenol Blue - 0.02 g
20 % Glycerol - 2 ml
200 mM betamercaptoethanol - 0.156 ml
Total volume was made to 10 ml with distilled water

Gel Composition

Materials	Stacking gel 4% (4ml)	Separating gel 10% (10ml)
H_2O	6.1 ml	4.1 ml
Acrylamide 30%	1.3 ml	3.3 ml
Separating gel buffer (Tris 1.5 M pH 8.8)	-	2.5 ml
Stacking gel buffer (Tris 1.5 m pH 6.8)	2.5 ml	-
SDS (10%)	100 µl	100 µl
APS 10%	50 µl	50 µl
TEMED	10 µl	5 µl

Gel Composition

Preparation of Gel

The two glass plates were clamped by the cassette holder. The components of the running gel were poured in between the glass plates. Water was poured on the top of the gel to remove the air bubbles and to have a smooth surface. The gel was allowed to polymerize. After polymerization the components of the stacking gel was added. The comb was inserted in the stacking gel layer without any bubbles. It was allowed to polymerize. After polymerization the comb was removed.

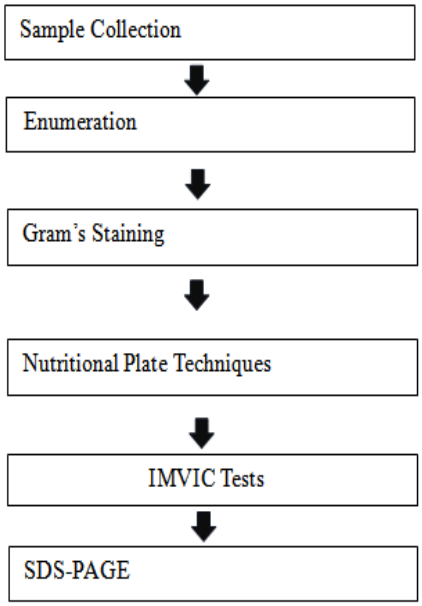
Preparation and Gel Electrophoresis of samples

The samples were mixed with 2:1 ratio of sample buffer and heated up to 90^0C for 3 minutes. Then it was cooled immediately. The samples were loaded in the gel and run in 50V till the samples crosses the stacking gel. Then current was raises to 100V. The electric field was turned off after 10 minutes of dye run out.

Coomassie brilliant blue staining

Following electrophoresis placed the gel in the Coomassie staining solution. The gel incubated for 6 hours to overnight in a staining solution. The gel was kept in a de-staining solution until the background was transparent. Then the bands were observed.

Methods



III. RESULT AND DISCUSSION:

Colony Forming Unit

The results indicate that both sheep and goat milk samples contain moderate to high levels of bacterial growth.

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Table

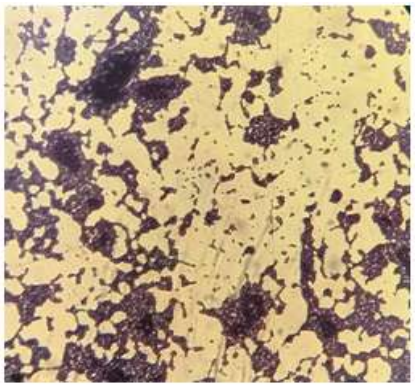
S. No	Name of the test samples	No. of bacterial colonies-24 hrs	The total volume of the test sample	CFU/ml/gm-24 hrs
		LB Agar		LB Agar
1.	Sheep milk	16	100 µl	0.16×10^{7}
2.	Goat milk	56	100 µl	0.56×10^{7}

Colony Forming Unit

Gram's Staining

The microbiological analysis of sheep and goat milk samples revealed a predominance of Gram-positive bacteria. Microscopic examination confirmed the presence of Gram-positive cocci and Gram-positive rod. This Gram-positive profile is desirable for probiotic applications and nutritional enrichment, underscoring the potential of these milk samples for developing value-added dairy products.

Goat milk (Gram-negative rod)



Sheep milk (Gram-positive cocci)

S.No	Name of the sample	Result of gram's staining	Motility
1.	Sheep milk	Gram-positive cocci	Non-motile
2.	Goat milk	Gram-negative rod	Non-motile

Gram's Staining Result

Selective Media

In the selective medias of Milk Agar Plate and Potato Dextrose Agar Plate the more bacterial strains grows in the Milk Agar Plate which showed that bacteria present in the Sheep and Goat milk sample has an ability to produce high efficiency protease enzyme.

IMVIC Test

Indole Test

The biochemical examination of the sheep and goat milk indicate that the positive result of Indole test which cursor the presence of tryptophanase enzyme. The detection of tryptophanase enzyme indicates a dynamic microbial ecosystem, showcasing the milk's natural

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richness and this enzyme widely used in industries. Formation of red colour ring indicates the positive result.

Indole test – Positive

Methyl Red Test

The microbiological analysis of the sheep and goat milk sample yielded a negative Methyl Red (MR) test result, indicating minimal acid production. The negative MR test result demonstrates compliance with dairy safety standards and underscores the dairy's commitment to quality. This outcome reassures consumers of the milk's safety and purity. The negative result indicates that the bacteria does not have an ability to perform mixed acid fermentation of glucose. No colour formation indicates the negative result.

Methyl Red Test – Negative

Vogas Proskauer Test

The Voges-Proskauer test detects acetoin, a metabolite produced by certain bacteria, indicating potential contamination. A negative result is generally desirable. This makes big drawback since acetoin is widely used in industrial level. The enzyme butyraldehyde dehydrogenase unavailability also confirmed. The formation of the diacetyl is also absent. No colour formation indicates the negative result.

Vogas Proskauer Test – Negative

Simmon's Citrate Utilization Test

The Biochemical test of Simmon's Citrate Utilization indicates that bacteria present in the sample of sheep and goat milk has an ability to consume the citrate. This citrate-positive result highlights the milk's robust nutritional profile and compliance with dairy quality standards. Citrate metabolism optimizes lactose fermentation. Breaks down citrate, producing diacetyl, acetoin, and carbon dioxide. Regulates pH levels, ensuring optimal fermentation conditions. Formation of green colour in medium indicated the consumption of citrate by the bacteria from sheep and goat milk.

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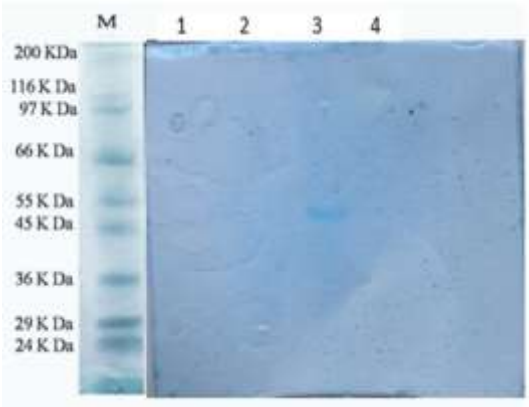
Simmon's Citrate Utilization Test-Positive

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE analysis of our sheep and goat milk sample yielded a positive result, revealing a rich protein profile that underscores the milk's exceptional quality. This outcome confirms the presence of essential nutrients, ensuring high-quality milk for various applications. The intact protein structure and effective processing suggest compliance with dairy industry standards, making our milk ideal for infant formula and dairy-based ingredients. This positive result reinforces our commitment to delivering premium milk products that meet the highest quality and nutritional standards. The presence of high-intensity protein bands in our sheep and goat milk sample has significant various implications.



IV. CONCLUSION:

The screening and identification of industrial enzyme-producing bacteria from sheep and goat milk have yielded promising results, revealing a diverse range of enzymes with potential applications in dairy processing, food production, and pharmaceuticals. This study highlights the untapped potential of sheep and goat milk as a source of novel enzymes, offering opportunities for improved efficiency, yield, and sustainability in various industries. Future research should focus on optimizing enzyme production, characterization, and scale-up, as well as exploring new applications and collaborations between academia, industry, and regulatory bodies. The exploration of sheep and goat milk's microbial enzymes not only expands our understanding of microbial diversity but also underscores the importance of preserving traditional dairy practices. By embracing this biodiversity, we can unlock novel solutions for sustainable development and improved food security. This research demonstrates the importance of interdisciplinary collaboration, bridging the gap between dairy science, microbiology, and biotechnology. Further investigation will unveil the full potential of these enzymes, driving innovation and economic growth.

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