ISOLATION, SCREENING AND CHARACTERIZATION OF MICROORGANSIM WITH FIBRINOLYTIC ACTIVITY FROM VARIOUS FERMENTED FOODS

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Isolation, Screening and Characterization of microorganism with Fibrinolytic Activity from various Fermented foods

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ABSTRACT: One of the major problems of causes of death worldwide is cardiovascular disease, according to the American heart association. An unwanted blood clot (thrombi) is caused by dysregulation in the blood clotting system caused by abnormalities in the blood that provoke thrombosis and lead to heart attack, strokes, blocking of blood vessels and coronary artery disease etc. An Increasing percentage of deaths due to thrombosis. Commonly treat thrombosis different thrombolysis agents are used. A huge potential has a thrombolytic agent and it plays a major role. The fibrinolytic enzyme is considered a potent thrombolytic agent to treat and prevent cardiovascular diseases (CVDs). The commercially available thrombolytic agents such as Urokinase plasminogen activator, Streptokinase plasminogen activator and tissue plasminogen activator are generally safe but are very expensive and are less effective. Thrombolytic agents are of two types, one is plasmin like proteins which directly degrade fibrin and another one is plasminogen activator which activates plasminogen into active plasmin to degrade fibrin. The molecular weights of the proteins that play roles in the fibrinolytic activity ranged from approximately 30 to 153 kDa. The health benefits of some fermented foods are a synthesis of nutrients, prevention of cancer and cardiovascular disease due to the presence of functional microorganisms, which possess probiotics properties and anticoagulant. The fermented foods as sources of microorganism used were Rice water, Dhokla, Idli water. The total positive number of isolates were 27, out of which only 7 had significant proteolytic activity for fibrinolysis.

KEYWORDS: cardiovascular disease, thrombolytic activity, fermented food, fibrinolytic activity, plasminogen.

1. INTRODUCTION:

Cardiovascular disorders are a major cause of death and morbidity worldwide. As per the American Heart Association prediction that, by 2030: death by the CVDs shall go over 23.6 million (Hidayati et al. 2021) (Gowthami and Madhuri n.d.). Thrombosis like vascular blockage, pulmonary embolism, peripheral occlusive disease, deep vein thrombosis, stroke and acute myocardial infraction due to blood clot (Nourah Hassan and Fareed Shawky 2020). tPA is a serine protease which catalyses the conversion of plasminogen to plasmin, a major enzyme responsible for breakdown of fibrin in the blood clots. Plasminogen activators, such as tPA, urokinase, alteplase and reteplase are used in the clinical medicine to treat embolic and thrombotic strokes (Stephani et al. 2017). There are therapeutic and surgery treatments also available but, the most effective drugs for treating thrombosis are fibrinolytic enzymes, which can be divided into two types according to their action mode: plasminogen activators (PAs), including tissue plasminogen activator (t-PA), streptokinase (SK, 3.4.99.22) and urokinase (UK, EC 3.4.21.31), which can lyse fibrin clots. Fibrin is the major protein component of blood clots, which are formed from fibrinogen by thrombin. Insoluble fibrin can be hydrolyzed to fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators (Oncom 2014). Despite extensive research about them and their wide use as thrombolytic agents, these PAs are high in price and low in specificity, prompting the researchers to explore for safer and cheaper resources. Microorganisms contribute to the production and use of the fibrinolytic enzymes since ancient times, and researchers have paid. Over the last 10 years, several effective thrombolytic agents have been identified and characterized from microorganisms, plant, fungi, earthworms, snake venoms, centipede venoms, insects and leeches (Anand et al. 2014) (Wang et al. 2006). The agents are of interest as useful tools for understanding fibrinolytic mechanism and as potential therapeutic drugs (Hu et al. 2019). Microorganisms are the most important and cheap source of fibrinolytic enzymes, and many of them, such as Streptokinase and Staphylokinase, which were isolated from *Streoptococcus hemolyticus* and *Streptococcus aureus*, are effective in thrombolytic therapy(Khusro et al. 2018). Also, consequently several lines of investigation are currently being pursued to enhance the efficacy and specificity of fibrinolytic therapy. Recently

fibrinolytic enzymes have been discovered from fermented food (Yoon n.d.) (Mine, Kwan Wong, and Jiang 2005).

2. MATERIAL AND METHOD:

Media and Reagents:

Agar agar powder (HiMedia, India), Nutrient Broth (SRL CHEM, India), Luria Bertani broth (Sisco laboratories, India), Casein Enzyme and Casein Hydrosylate (Chemdyes corporation, India), Simmons Citrate Agar (HiMedia, India), Methyl Red (HiMedia, India), Vogues Proskauer (HiMedia, India), MSA Media (HiMedia, India), Starch Hydrolysis media (Sisco laboratories, India), Peptone (HiMedia, India), Nitrate broth (HiMedia, India), were used.

2.1 Sample Collection:

The various sources for collection of microbes selected were: Rice water, Fermented food water, Marine water and Milk. Marine soil and marine water samples were collected from the Madhavpur and Dwarka, Gujarat. Samples were collected in sterile polythene bags. Milk sample were collected from bulk tank milk. Rice water, dhokla, idli samples were collected from kalawad road area.

2.2 Isolation of Fibrinolytic Enzyme Producing strain:

The serially diluted samples, 1 mL each, was collected and spread onto the surface of skimmed milk agar (SMA) plate (Hi Media, India) with the composition of bacteriological agar and were incubated at 37 °C for 24 h (Syahbanu et al. 2020). Bacterial strains with proteolytic potential were screened and separated based on the clear zone formed in the SMA. (Nourah Hassan and Fareed Shawky 2020) (Anusree et al. 2020). Skim milk agar plate method was performed for determining casienolytic activity along with thrombolysis activity of the microorganisms. Those microorganisms were selected for further screening tests, which showed halo zone of hydrolysis of casein protein present in the milk.

2.3 Screening of fibrinolytic enzyme:

The phenotypic assay included the following 2 tests: casein Hydrolysis test and heated plasma agar plate method. Different methods including haemolysis on blood agar (blood haemolysis activity by formation of beta-haemolytic ring), heated plasma agar (for plasmolytic

activity), casein agar and skim milk agar plates (for casienolytic activity) along with determining thrombolysis activity were performed to evaluate staphylokinase activity for checking fibrinolytic activity (Nourah Hassan and Fareed Shawky 2020) (Bin et al. 2013) (Sevinc and Demirkan 2011).

2.3.1 Caseinolytic activity:

The casein hydrolysis test was performed by making wells in the casein agar media plates, adding 2.0 microlitres of isolates into the wells. The observation of zone of hydrolysis was measured and interpreted (Bin et al. 2013).

2.3.2Plasmolytic activity:

The heated plasma agar was performed to check the plasmolytic activity of the enzyme. The isolates were added into the wells, 2.0 microlitres. The heated plasma test was performed by making wells and The observation of clear halo zone measured and interpreted (Kaya-Ongoto et al. 2019).

2.4 Morphological identification:

The bacterial isolates with thrombolytic and fibrinolytic activity were then identified morphologically using various staining and biochemical tests (Priskila et al. 2022). The smear was prepared from isolated cultures on clean, grease free glass slides and stained with Gram's Staining method.

2.5 Biochemical identification:

The biochemical tests performed were for the confirmation of the *Staphylococcus aureus* by: Catalase test, KOH test, Indole test, Urease test, Simmon's citrate test and Nitrate reduction test (Thaker, Brahmbhatt, and Nayak 2013).

2.6 Antibiogram pattern:

Antibiogram pattern of the isolated S. aureus to some antimicrobial agents. The susceptibility of isolates to different antimicrobial agents was done by disk diffusion method using commercial disks purchased from Hi Media PVT LTD. Antibiotics discs were used for the antibiotic susceptibility test performed, which were: Gentamicin (10 mcg), Streptomycin (25 mcg), Vancomycin (30 mcg), Kanamycin (30 mcg), Neomycin (30 mcg), Ofloxacin (2 mcg), Rifampicin (15 mcg), Erythromycin (15 mcg),Ceftazidime (30 mcg), Chloramphenicol (25 mcg), Novobiocin (30 mcg) and Oxy-Tetra (Imipenem) (10 mcg). The results of the

sensitivity of microorganism towards the antibiotics was observed after 24 h of incubation.The highest sensitivity was found from Gentamicin antibiotic test and the lowest sensitivity was found from Rifampicin antibiotics test.(Javed et al. 2021)

3. RESULT AND DISSCUSION:

3.1 isolation of Fibrinolytic Enzyme producing bacteria:

Analysis of results revealed that the isolation of fibrinolytic producing microorganism, the serially dilutes samples were spread on SMA plates and the colonies showing zone of hydrolysis (Figure: 1) were selected for further screening tests.

3.2 Screening of fibrinolytic enzyme producing microorganisms:

Phenotypic assays including: Casein Hydrolysis and Heated Plasma Agar assay was performed. The isolates of microorganism showed caseinolytic activity by formation of significant zones of hydrolysis of casein observed.

3.2.1 Casein Hydrolysis Activity

In casein hydrolysis assay, the hydrolysis of casein was identified by a zone of hydrolysis around the growth of colonies on casein agar plates. Only 20 isolates showed clear halo zones around the colonies. A halo zone of 11 to 15 mm was measured in 10 isolates, 16 to 19 in 6 isolates and in 3 isolates a zone of 20 to 21 mm was observed. The highest zone measuring about 23 mm was observed only in single isolate. (Figure: 2)

3.2.2 Proteolytic Activity

After inoculation of fibrinolytic enzyme producing *S. aureus* on plasma agar plate using the well diffusion technique a clear fibrinolytic halo zones was observed around the well after overnight incubation at 37°C. Halo zones were observed in 7 *S. aureus* isolates, indicating their ability to produce fibrinolytic enzyme. (Figure: 3)

3.3 Morphological and Biochemical characterization

The colony morphology was examined and they were identified as cocci and gram staining results revealed that the bacterial species to be a gram positive. All isolates were Gram positive Bacilli in chain. (Figure: 4)

Table-1. Morphological and culture characteristics of *S. aureus*		
Gram staining	**Shape and colour**	**Culture characteristics on SMA media**
Gram positive bacilli (in chain)	Rod shape, purple colour	SMA: off white colony with zone of hydrolysis

In the biochemical tests, the results were: in urease test (16 positive results), in catalase test (8 positive results), in H_2S test (12 positive results), in methyl red test (16 positive results), in vogues proskauer test (15 positive results) and in citrate test (17 positive results) found. Among the 27 isolates, only 7 strains exhibited significant proteolytic activity and fibrinolytic activity.

3.4 Antibiogram Pattern:

In the study of isolates were found variably resistant to the antibiotics tested. The isolates showed highest sensitivity towards Gentamycin and the lowest sensitivity towards the Rifampicin. (Figure: 5)

Antibiotic Name	**Zone of inhibition (in mm)**				
	SA 3 (mm)	**SA 9 (mm)**	**SA 11 (mm)**	**SA 23 (mm)**	**SA 24 (mm)**
Kanamycin	15	13	18	15	14
Rifampicin	9	9	8	6	8
Novobiocin	13	12	12	10	10
Neomycin	18	16	18	15	16
Chloramphenicol	15	11	21	15	17
Vancomycin	13	11	11	10	10
Erythromycin	21	15	12	15	20
Gentamicin	22	19	13	13	17
Streptomycin	15	16	15	14	17
Oflaxacin	18	19	14	15	18
Ceftazidime	-	-	-	6	10
Imipenem	32	35	25	30	35

CONCLUSION:

Collection of samples from various sources, Marine soul & Marine water was collected from Dwarka, Gujarat. There were a Milk sample, Rice water & some of the fermented food samples (Dhokla & Idli) collected from Kalawad road, Rajkot. Serially diluted samples spread on Skim milk agar plate and the colonies shifted to LB plate. Then the bacterial strain was culture into nutrient broth, protease activities of the isolates were determined by standard Casienolytic

assay and Heated Plasma Agar assay. Casienolytic activity was assayed by using Casein plate and this diffusion technique was used to check the fibrinolytic enzymes activity. In heated plasma agar it was mixed with nutrient agar and plated. This diffusion technique was used to check the proteolytic activity of enzyme. Following biochemical tests were performed: Urease test, Catalase test, H_2S, Methyl Red (MR) test, Vogues Proskauer (VP) test, Nitrate Reduction test, KOH test, Indole test and Simmon's Citrate test. In the heated Plasma Agar assay clear halo zones found. To check the antibiotic susceptibility of the microorganism (sensitivity) was performed by using antibiotic discs. The antibiotics used were: Kanamycin, Rifampicin, Novobiocin, Neomycin, Chloramphenicol, Vancomycin, Erythromycin, Gentamycin, Streptomycin, Ofloxacin, Ceftazidime, Imipenem.

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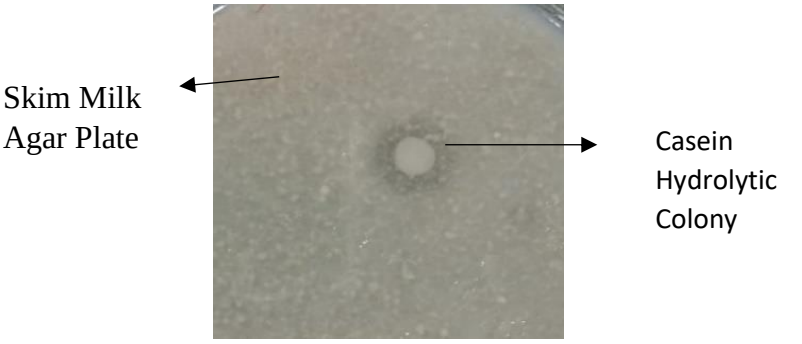

Figure- 1: Halo zone around Colony on Skim Milk Agar Plate

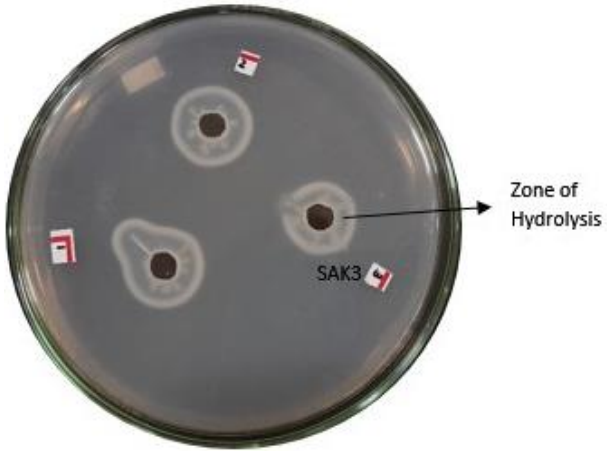

Figure- 2 : Zone of Hydrolysis on Casein agar plate

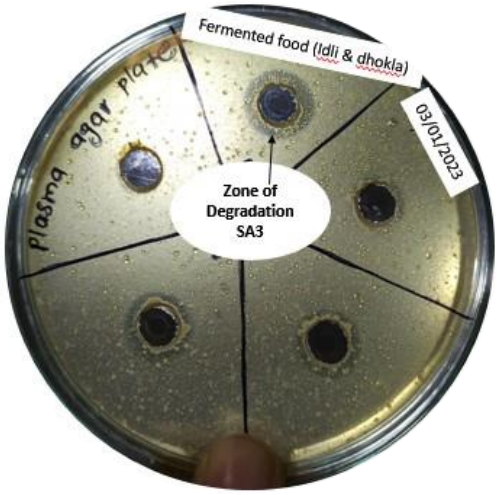

Figure- 3: Zone Degradation on Plasma agar plate

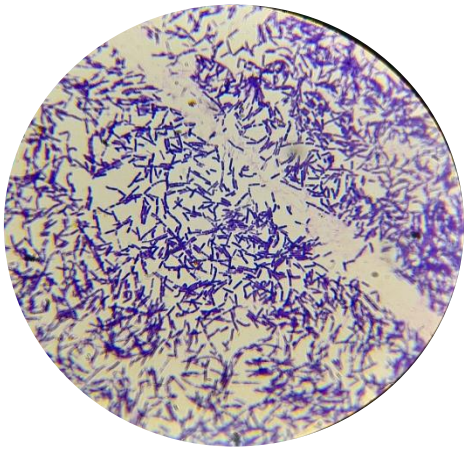

Figure- 4: Gram Positive, Purple colored, rod shape organism were seen

Figure- 5: Antibiotic Sensitivity