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Isolation and Purification of Fibrinolytic Enzyme from Microbial Source

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ABSTRACT: Blood is a bodily fluid and a connective tissue comprising of different types of cells, also called as corpuscles, these are erythrocytes (red blood corpuscles or RBC), leukocytes (white blood corpuscles or WBC), and thrombocytes suspended in a liquid medium called as Plasma. When human body loses blood through a minor wound or injury, platelets cause the blood to clot so that the bleeding stops. Blood clotting, or coagulation, is an important process that prevents excessive bleeding when a blood vessel is injured. Coagulation is the process by which blood changes from a liquid to a gel clot or fibrin. It potentially results in haemostasis, the cessation of blood loss from a damaged vessel, followed by repair. Sometimes, however, clots are formed inside the vessels without an obvious injury or do not dissolve the clots naturally. This is known as hypercoagulable state or thrombophilia. In humans, decreased blood flow or stasis seems to be the dominant component resulting in thrombophilia. In primary screening all three site culture showed positive result but in secondary screening only Slaughter House at Nigdi (SHC), Fish Market at Pimpri (PFMS). 16s rRNA Sequencing analysis and phylogenetic bacterial species isolated from Nigdi Slaughter House shared closest homology with *Bacillus subtilis* strain DSM 10. Bacterial species isolated from Pimpri Fish Market shared closest homology with *Pseudomonas baetica* strain a390T. The present study gives the information that *Bacillus subtilis* and *Pseudomonas baetica*like bacterial species are one of the potent sources for the fibrinolytic enzyme. *Pseudomonas baetica*is studied for the first time as a source of fibrinolytic enzyme.

KEYWORDS: Enzyme, blood clots, coagulation, baetica, RBC, WBC.

I. INTRODUCTION

Blood is a bodily fluid and a connective tissue comprising of different types of cells, also called as corpuscles, these are erythrocytes (red blood corpuscles or RBC), leukocytes (white blood corpuscles or WBC), and thrombocytes (platelets) suspended in a liquid medium called as Plasma. Red blood cells transport oxygen from the lungs to all of the living tissues of the body and carry away carbon dioxide. White blood cells play important roles in immune system for example antigen determination, antigen presenting, messengers and antibody producers. Platelets are cells without nuclei, which work with blood clotting factors at the site of wound. They adhere to the walls of blood vessels, thereby plugging the rupture in the vascular wall. Plasma is relatively clear, yellow tinted fluid consisting of approximately 92% of water. Plasma contains blood clotting factors, vitamins, minerals, hormones, enzymes, antibodies, and other proteins which are required for the smooth functioning.

When human body loses blood through a minor wound or injury, platelets cause the blood to clot so that the bleeding stops. Blood clotting, or coagulation, is an important process that prevents excessive bleeding when a blood vessel is injured. Coagulation is the process by which blood changes from a liquid to a gel clot or fibrin. It potentially results in hemostasis, the cessation of blood loss from a damaged vessel, followed by repair. The mechanism of coagulation involves activation, adhesion, and aggregation of platelets along with deposition and maturation of fibrin. Coagulation is highly conserved throughout biology; in all mammals, coagulation involves both a cellular (platelet) and a protein (coagulation factor) component. The system in humans has been the most extensively researched and is the best understood.

Sometimes, however, clots are formed inside the vessels without an obvious injury or do not dissolve the clots naturally. This is known as hypercoagulable state or thrombophilia. In humans, decreased blood flow or stasis seems to be the dominant component resulting in thrombophilia[(1).] Decreased fluidity of the blood in the affected vessel results in

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the pathological thrombi which occlude the blood flow and leads to number of disease conditions like superficial phlebitis, Deep vein thrombosis, Pulmonary embolism, Hepatic vein thrombosis, Renal vein thrombosis, Brain stroke and Cardiovascular disease[1, 2].

Superficial phlebitis is one of commonly occurring disease condition approximately 1 per 1000 cases, which have characteristic inflammatory thrombotic process in superficial vein. The people diagnosed with superficial phlebitis are 6% to 44% associated with deep vein thrombosis where 20% to 33% people are associated with the asymptomatic pulmonary embolism and 2% to 13% with the symptomatic pulmonary embolism [3]. Common symptoms in response to hypercoagulable state include pain in legs, tenderness along the vein, warm, erythema (reddening of the skin), and edema (swelling of the tissue).

Venous thromboembolism is a collective term used for deep vein thrombosis of lower extremities and pulmonary embolism. It is the emobolisation of venous thrombi to the pulmonary circulation. On an average 50 per 100,000 patients of Deep vein thrombosis and 70 per 100,000 patients of pulmonary embolism with or without incidence of deep vein thrombosis [4].

Hepatic vein thrombosis also known as **Budd- Chiari** syndrome is rare disorder characterized by the obstruction of the blood out flow from the liver. This results in the intense pronounced congestion around the terminal hepatic venules, cell necrosis, Scant, inflammatory reaction, hepatomegaly, right upper quadrant abdominal pain and ascites. Predisposing factor include age, child- Pugh score, response of ascites to diuretics and use of oral contraceptive agents increases the risk of hepatic vein thrombosis[5].

II. MATERIALS AND METHODS

1. Sample Collection

100 ml Samples were collected in sterile bottles from three different sites for the isolation of prospective fibrinolytic bacteria. These Sites were Slaughter House at Nigdi, Pune, Fish Market at Pimpri, Pune and River at Ravet, Pune.

2. Primary Screening

All three samples were processed separately. Serial dilution each sample was done with physiological saline upto 10^{-3} and 0.1ml of dilution was plated on nutrient agar plates and incubated at 37˚C for 24 hours for microbial growth. Each well isolated bacterial colony after 24 hours of growth on nutrient agar plate was observed for colony appearance; shape, elevation, edge, optical characteristics, consistency, colony surface and pigmentation. Colonies with different morphology were recorded and spot inoculated on Skim milk agar plates for analyzingproteolytic activity [46]. Cultures showing zone of clearance on Skim milk agar plates were further selected for Secondary Screening.

3. Secondary Screening

Each culture selected after primary screening were spot inoculated on fibrin agar plate for 24 hours at 37°C [47]. Zone of clearance was noted after incubation for each culture.

4. Morphological and Biochemical characterization

Isolated cultures showing larger zone of clearance on fibrin agar plate were identified by morphological and biochemical characterization [48]. Various tests
include –

.4.*1.* Colony Morphology

Each well isolated bacterial colonies after 24 hours of growth on nutrient agar plate was observed for colony appearance; shape, elevation, edge, optical characteristics, consistency, colony surface and pigmentation.

4.2. Gram staining techniques

A thin smear of each of the pure 24 h old culture was prepared on clean grease-free slides, fixed by passing over gentle flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 sec and rinsed with water. The smear were again flooded with Lugol's iodine for 30 sec and rinsed with water, decolourized with 70% alcohol for 15 second and were rinsed with distilled water. They were then counter stained with 2 drops of Safranin for

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60 sec and finally rinsed with water, then allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram-negative cells appeared pink or red while gram-positive organisms appeared purple.

4.3. Spore staining technique

This test is to detect the presence of bacteria endospores. Heat-fixed smears of the organisms. Samples were prepared on separate slides and flooded with 5% Malachite green solution and steamed for a minute. The stain was washed off with water and counter stained with 2 drops of Safranin solutions for 20 seconds. The slides were allowed to air dry and examined under oil immersion objective (100) lens. Endospores stained green while vegetative cells stained pink.

4.4. Motility test

A sterile needle was used to pick a loop of a 24 hours old culture and was stabbed onto nutrient agar in glass vials. The vials were incubated at 37°C for 24-48 hours. Non-motile bacteria had growth confined to the stab line with definite margins without spreading to surroundings area while motile bacteria gave diffused growth extending from the surface.

4.5. Catalase test

A small quantity of 24 h old culture was transferred into a drop of 3% Hydrogen peroxide solution on a clean slide with the aid of sterile inoculating loop. Gas seen as white froth indicates the presence of catalase enzyme.

4.6. Coagulase test

Coagulase is an enzyme capable of coagulating certain blood plasma, notably human and rabbit plasma. This test differentiates pathogenic from non-pathogenic Staphylococcus spp., the test was carried out using 18-24 h old culture. A loopful of isolated bacterium was emulsified with normal saline solution on a microscope slide. A drop of undiluted plasma was added to the suspension and stirred for five seconds. A coagulase positive result was indicated by clumping of colonies together.

4.7. Methyl red test

Five millimeters of glucose phosphate broth were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 hours. At the end of incubation, few drops of methyl red solution were added to each test and colour change was observed. A red colour indicates a positive reaction.

4.8. Voges-proskaeur test

Five millimeter of glucose phosphate broth were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 hours. After incubation, 6% α-naphhtol and 6% Sodium hydroxide were added to about 1 mL of the broth culture. A strong red colouration formed within 30 min indicates positive reaction.

4.9. Indole test

Tryptone broth (5 mL) was placed into different test tubes after which a loopful of the bacterial isolates was inoculated into the test tubes, leaving one of the test tubes uninoculated to serve as control. The test tubes were then incubated at 37°C for 48 hours. After incubation, 0.5 mL of Kovac's reagent was added and shaken gently; it was allowed to stand for 20 minutes to permit the reagent to rise. A red or red-violet colour at the top surface of the tube indicates a positive result while yellow colouration indicates a negative result.

4.10. Starch hydrolysis

This is used to assay the ability of microorganisms that can produce enzymes that degrade substrate with carbon compounds. Nutrient agar was prepared with 1% soluble starch and was sterilized. The medium was poured into sterile plates and was inoculated by streaking the organisms once across the plates after solidifying. The plates were incubated at 37°C for 24 hours after which they were flooded with Gram's iodine. Unhydrolysed starch forms a blue colour with the iodine. Hydrolysed starch appears as a clear zone due to alpha amylase activity while reddish brown zones around the colony indicates partial hydrolysis of starch (to dextrans).

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4.11. Citrate test

This test detects the ability of an organism to use citrate as a sole source of carbon and energy. About 2.4 g of citrate agar was dissolve in 100 mL of distilled water. About ten milliliter (10 mL) of citrate medium was dispensed into each tubes and covered, then sterilized and allowed to cool in a slanted position. The tubes were inoculated by streaking the organisms once across the surface. A change from green to blue indicates utilization of the citrate.

4.12. Oxidase test

A piece of filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick a colony of the test organism and smeared on the filter paper. If the organism is oxidase producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour.

4.13. Sugar fermentation

Sugar fermentation test was carried out to determine the ability of organisms to ferment sugars with production of acid and gas. Sugar indicator broth was prepared using peptone water medium containing 1% fermentable sugar and 0.01% phenol red. About ten milliliters of sugar broth was dispensed into each of the test tubes, durham tube which would trap the gas if produced was inverted carefully. The test tubes were autoclaved and inoculated with a loopful of 24 hours old culture of the test organisms after then incubated for 2-7 days at 36±1EC and observed daily for acid and gas production. Yellow colouration indicates acid production while gas production was indicated by displacement of the medium in the durham tube.

III. RESULTS

Three different sites in Pune were selected for the collection of samples to isolate microbial species showing fibrinolytic activity. Approximately 100ml of sample in sterile collection bottles was collected from each site. These Sites were Slaughter House at Nigdi, Pune, Fish Market at Pimpri, Pune and River at Ravet, Pune.Figure 1 and 2 shows the Bacterial cultures SHC and PFMS showing larger zone of clearance on fibrin agar plate were identified by morphological and biochemical characterization. Bacterial culture SHC belonged to Bacillus Spp. while bacterial culture PFMS belonged to Pseudomonas Spp. Figure 3 and 4 shows the Bacterial cultures SHC (isolated from Nigdi Slaughter House) and PFMS (isolated from Pimpri Fish Market) showed activity on Fibrin Agar Plates. While bacterial culture RS isolated from Ravet river did not show activity on Fibrin Agar Plate.

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Fig.1. Bacterial culture (SHC)

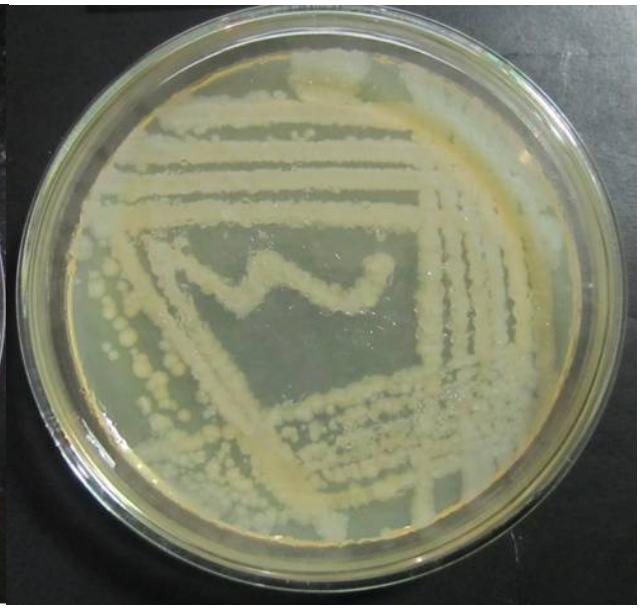

Fig. 2. Bacterial culture (PFMS)

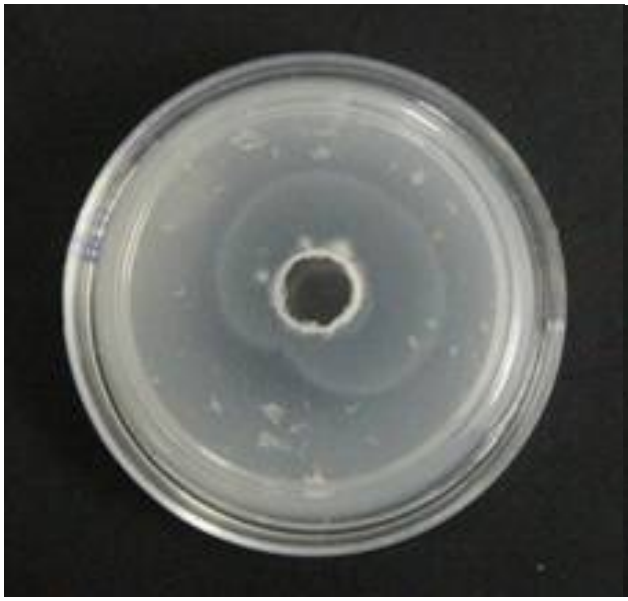

Fig. 3.Partially purified enzyme from SHC

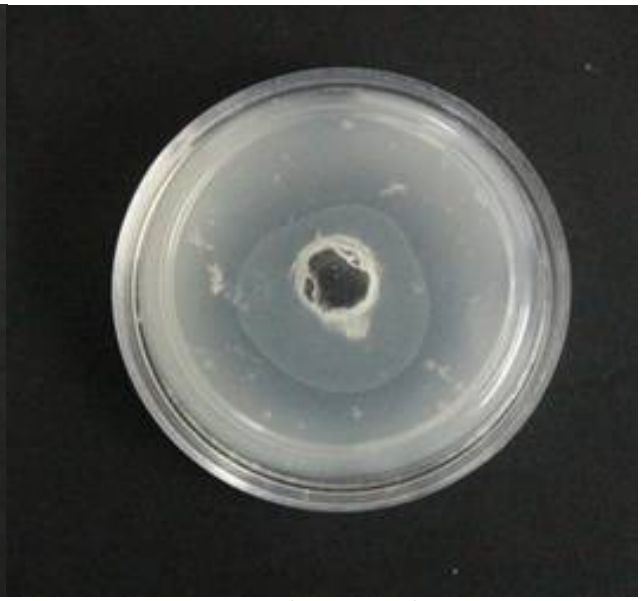

Fig 4.Partially purified enzyme from PFMS

IV. CONCLUSION AND FUTURE WORK

The study describes the ability of *Bacillus subtilis* and *Pseudomonas baetica*to produce fibrinolytic enzyme for the application as a thrombolytic agent. Microorganisms are the important sources of thrombolytic enzymes with the fast growth and easy control characteristics. Microorganisms can be manually controlled to obtain the target product.

In the conclusion, we can say, the present study gives the information that *Bacillus subtilis* and *Pseudomonas baetica*like bacterial species are one of the potent sources for the fibrinolytic enzyme. *Pseudomonas baetica*is studied for the first time as a source of fibrinolytic enzyme. This novel strain of *Pseudomonas baetica* is reported as fish

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pathogen recently in 2012. Its further molecular study and in vitro and in vivo analysis may give us further details to exploit the enzymes in commercial application. Finally, from the present study it clearly indicates that this fibrinolytic enzymes can also be used as a clinical therapeutic agent that cure the thrombosis and related dysfunctions after further study.

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