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Isolation, production and application of fibrinolytic enzyme from fermented rice, pulse and groundnut.

Aislamiento, producción y aplicación de enzima fibrinolítica a partir de arroz, legumbres y maní fermentados

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ABSTRACT

Accumulation of fibrin in blood vessels usually results in thrombosis, leading to myocardial infarction and other cardiovascular disease. For Thrombolytic therapy, microbial fibrinolytic enzymes have now attracted much more attention than typical thrombolytic agent because of the expensive prices and undesirable side effects of the latter. The fibrinolytic enzymes were successively discovered from different microorganisms. The most important among which is the Genus Bacillus from traditional fermented food. The physiochemical properties of these enzymes have been further identified. Therefore microbial fibrinolytic enzymes, especially those from food grade micro-organism, have the potential to be developed as functional food additive and drugs to prevent or cure other related diseases. In order to obtain Bacillus species producing fibrinolytic enzymes, the fermented food sample such as sprouted grain and processed grain were used. The heat tolerant isolates initially were selected for catalase test. Fibrinolytic activity of the selected isolates was determined by using Fibrin plate assay. From the above work, it can be concluded that the fibrinolytic enzyme produced by Bacillus from fermented food samples had the ability to degrade the fibrin and hence can be used for functional food formulation.

Keywords: Fibrin, myocardial infarction, thrombolytic agent, Bacillus, Fibrinolytic activity.

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RESUMEN

La acumulación de fibrina en los vasos sanguíneos suele provocar trombosis, lo que lleva a un infarto de miocardio y otras enfermedades cardiovasculares. Para la terapia trombolítica, las enzimas fibrinolíticas microbianas ahora han atraído mucha más atención que el agente trombolítico típico debido a los altos precios y los efectos secundarios indeseables de este último. Las enzimas fibrinolíticas fueron descubiertas sucesivamente a partir de diferentes microorganismos. El más importante entre los cuales es el género *Bacillus* de alimentos fermentados tradicionales. Las propiedades fisicoquímicas de estas enzimas se han identificado más. Por lo tanto, las enzimas fibrinolíticas microbianas, especialmente las de microorganismos de calidad alimentaria, tienen el potencial de desarrollarse como aditivos alimentarios funcionales y fármacos para prevenir o curar otras enfermedades relacionadas. Para obtener especies de *Bacillus* productoras de enzimas fibrinolíticas, se utilizaron muestras de alimentos fermentados tales como granos germinados y granos procesados, etc. Los aislados tolerantes al calor inicialmente se seleccionaron para la prueba de catalasa. La actividad fibrinolítica de los aislados seleccionados se determinó mediante el ensayo de placa de fibrina. Del trabajo anterior, se puede concluir que la enzima fibrinolítica producida por Bacillus a partir de muestras de alimentos fermentados tenía la capacidad de degradar la fibrina y, por lo tanto, se puede usar para la formulación de alimentos funcionales.

Palabras clave: fibrina, infarto de miocardio, agente trombolítico, bacilo, actividad fibrinolítica.

INTRODUCTION

Bacillus: Bacillus are rod shaped gram positive endospores forming bacteria. Endospores very resistant to many adverse conditions formed not more than one per cell. Endospores are usually cylindrical or oval or round shape it can be aerobic or facultative anaerobic during the formation of endospore mother cell may not change its shape. Catalase formed by most species but not all. The peptidoglycon of most species belongs to the directly cross-linked meso-diaminopimelic acid the main isoprenoid quinone is menaquinone with seven isoprene units. The phospholipids that occur most commonly is phosphatidylethanolamine and phosphatidylglycerol, several Bacillus species produces carbohydrate capsules. Bacillus anthracis produced a poly-gama-D-glutamyl capsule in vivo; most Bacillus species are mobile by means of peritrichous flagella. Bacillus anthracis is normally non-motile and Bacillus cereus is normally motile. Bacillus subtilis is one of the best understood prokaryotes, in terms of molecular biology and cell biology. Many Bacillus species

cause diseases, Bacillus anthracis cause anthrax and Bacillus cereus cause food borne illness, Bacillus thuringiensis used as insecticide, Bacillus subtilis used as important model organism, it also spoil the food material.

Fibrin: Fibrin (also called Factor) is a fibrous, non-globular protein involved in the clotting of blood. It is a fibrillar protein that is polymerized to form a "mesh" and forms a haemostatic plug or clot (in conjunction with platelets) over a wound site. Fibrin is involved in signal transduction, blood coagulation, platelet activation and protein polymerization.

Fibrinolytic enzyme: The enzyme which dissolve the blood clot from the blood vessel is called fibrinolytic enzyme now a day's different type of fibrinolytic enzyme are obtained from the different microorganism, some of them are- nattokinase, streptokinase etc.

Streptokinase: Streptokinase is produced by various strain of beta-hemolytic Streptococci, the enzyme single chain polypeptide that exert its fibrinolytic action indirectly by activating the circulatory plasminogen, the complete amino acid sequence of streptokinase was first established by Jackson and tang (1982), its molecular mass is 47kda. Streptokinase is made up of 414 amino acids.

Nattokinase: Nattokinase is the first fibrinolytic enzyme, discovered in Japan from traditional fermented food, called natto. Nattokinase is produced by fermentation process by adding Bacillus natto, a beneficial bacteria boiled soya bean. The resulting Nattokinase enzyme, is produced when Bacillus natto acts on the soybeans. While other soy foods contain enzymes, it is only the natto preparation that contains the specific Nattokinase enzyme. Natto is a fermented cheese-like food that has been used in Japan for over 1000 years for its popular taste and as a folk remedy for heart and vascular disease.

In recent researches we have found that according to the WHO 17 million people die every year due to cardiovascular diseases. Here, microorganisms such as streptokinase from Streptococcus hemolyticus and Staphylokinase from Strapthylococcus aureus are important resources that are mostly effective in thrombolytic therapy said by Colleen and Lijnen (1994). Development of other microbial fibrinolytic enzymes is still ongoing and much work needs to be done intensively and extensively, especially concerning thrombolytic effect in-vivo. While in process of research we came to know that fibrin is used in animal model, in-vitro functioning & cell-culture cultivation process.

In this study the fermented food sources of rice, pulses (pigeon pea) and groundnut are used to isolate bacterial species of bacillus subtilis by heat treatment and ethanol treatment that are leads to purification, characterization and production of these fermented

food sources with specific optimization of the species on basis of their production parameters such as temperature, pH, etc. The degradation of the fibrin into the fermented food sources which contributes to the production of the fibrinolytic enzyme, known catalysts for the biochemical reactions are helpful in blood clotting and also other thrombolytic agents are helpful in digestive system. Hence, the inappropriate clotting leads to the risk of cardiovascular diseases like myocardial infarction and stroke.

MATERIAL CHARACTERIZATION

A. Isolation of bacterial cultures from fermented samples.
B. Bacterial source: Pulses, Rice, Ground nut samples. The samples were obtained from different sources.
C. Isolation and Screening for heat resistance Bacillus.
D. Biochemical Characteristic of isolated culture.

EXPERIMENTAL ANALYSIS

A. Indole production test-

Tryptophan, an essential amino acid is oxidized by some bacteria by the enzyme Tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The indole test is performed by inoculating a bacterium into tryptophan broth. The indole produced during the reaction is detected by adding "Kovac's reagent" (dimethyl amino benzaldehyde) that produces a cherry red compound.

Procedure: The test organism was inoculated in peptone broth and an uninoculated tube was taken as control. The tubes were incubated at 37°C for 24 hrs. After incubation, 0.2 ml of Kovac's reagent was added for 5ml culture. Development of a cherry (deep) red colour layer in the top of the tube indicates positive test for indole production. Absence of the red colour indicates indole negative test.

B. Citrate utilization test-

Citrate test is used to differentiate bacteria on the basis of their ability to ferment citrate as the sole carbon source. The utilization of citrate depends on the presence of an enzyme citrase produced by the organisms, which breaks the citrate to oxaloacetic acid and acetic acid. These products are later converted to pyruvic acid and CO2 as shown below:

Citric acid $\longrightarrow$ Oxaloacetic acid + acetic acid $\longrightarrow$ Pyruvic acid + CO_2

$CO_2 + 2Na^+ + H_2O \longrightarrow Na_2CO3$ (alkaline)

(Bromothymol blue) (Persian blue colour)

Bromothymol blue is green in colour when acidic (pH 6.8 and below) and blue when alkaline (pH 7.6 and higher).

Procedure: SC agar was prepared and the test organisms was inoculate, the uninoculated tube serve as control. The tubes were incubated at 37°C for 96 hrs. The change in the color of the medium from green to Prussian blue indicates the positive test where as no change in color of the medium indicates negative test.

C. Physiological tests:

1. Growth at different temperatures:

Methodology:

- Series of test tubes, each having 5ml of Nutrient broth were inoculated with 50µl 16hrs old culture separately.
- The inoculated set of culture tubes were kept at different incubation temperature such as 5°, 10°, 30°, 40°, 50°C. The growth was monitored after 24hrs to 48hrs and ability of the culture to grow at different temperature were analyzed as indicated by the turbidity change.

2. Growth in presence of lysozyme:

Methodology:

- Series of test tubes, each having 5ml of Nutrient broth were inoculated lysozyme (0.001%) and then 50µl of 16hrs grown cultures were inoculated.
- The inoculated set of culture tubes were inoculated with Lysozyme enzyme (0.001%). The growth was monitored after 24hrs to 48hrs and the ability of grow in presence of lysozyme which was analyzed as indicated by the turbidity change.

3. Protein estimation: (by Bradford method): Preparation of protein standard (using BSA-Bovine Serum Albumin)

Materials:

- Stock solution (BSA) 1mg/ml.
- Clean and dry test tubes were taken and different concentrations of standard BSA solution are taken, such as 0-80microgram. Volume is made upto 500µL. 1mL of the Bradford reagent is added. After 10min of dark incubation take OD.
- OD was read at 660nm.

- Standard graph was plotted by taking OD on Y-axis and concentration of protein on X-axis

4. Fibrin plate method:

Materials: Fibrinolytic activity was determined by fibrin plate method and human plasmin was taken as positive control.

Methodology:

- Fibrin plate was prepared by adding the 5mg of human. Fibrinogen in 7ml of 0.1M barbital buffer, 10U of thrombin and 7ml of 10gm of agarose in a petri dish.
- This petri dish was heated at 80°C for 30mins to destroy the other fibrinolytic factors.
- To observe fibrinolytic activity of the enzymes, 10microleter of enzyme solution was dropped in fibrin plate.
- Plate was incubated at 37°C for 12hrs to18hrs. The activity of fibrinolytic enzyme was determined by the presence of clear zone formed.

RESULTS

Different fermented food sample such as Pulse powder, rice powder, Ground nut powder etc. were taken for the present study. Then samples were serially diluted with peptone water, after dilution plated on NA plates and incubated at 37°C for 24 hours and the total numbers of colonies were counted. For heat resistant isolates, sample was subjected to 80°C for 20mins and heat resistant colonies were counted.

In indole production test the development of a cherry red color layer in the top of the tube indicates positive test for indole production & absence of the red color indicates indole negative test which is done for obtaining a result of gram positive and gram negative organisms.

In citrate utilization test change in the color of the medium from green to Prussian blue indicates the positive test where as no change in color of the medium indicates negative test that shows the acidity and alkalinity level in the organisms.

Table 1: Total bacterial count and heat resistant count from fermented food sample:

Table 2: Catalase test: (+) positive reaction and (-) negative reaction all isolates are catalase positive:

SL.NO	Culture code	Source	Catalase activity
1	1	Gram nut	+
2	2	Arhar dal	+
3	3	Rice	+

Fig. 1: Showing catalase reaction

Table 3: Casein hydroysis by isolated culture: all isolates formed clear zone that shows its protease activity:

SL.NO	Code	Culture	Clear zone
1	1	1	No zone
2	2	2	Clear zone
3	3	3	Clear zone

Fig.2: Casein hydrolysis test

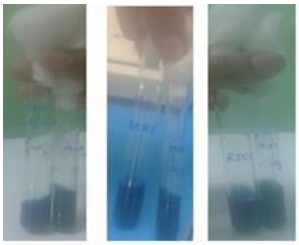

Fig.3: Sugar utilization test

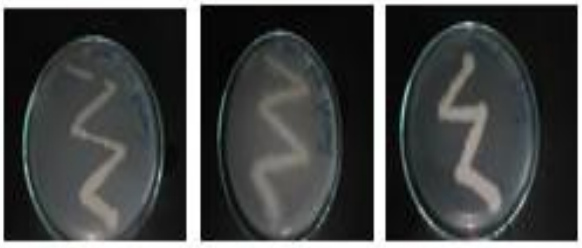

Fig. 4: Citrate utilization test

Fig. 5: Indole test

Fig. 6: Starch hydrolysis test

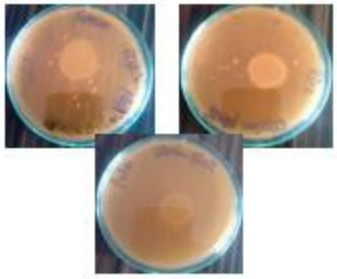

Fig. 7: Fibrin plate count grown in lb broth for 24hrs and supernatant was spotted on a fibrin plate and incubate at 37°c for 12-18hrs plasmin serveas positive control

Table 4: Growth at different temperatures

Growth at different temperature	Gram	Pulse	Rice
5°C	-	-	-
10°C	-	+	-
30°C	+	+	+
40°C	+	+	+
50°C	+	+	+
60°C	-	-	-

Table 5: Test sample

Sample	OD	Concentration of protein µg/ml
Rice	0.30	47
Gram	0.36	56
Pulse	0.52	82

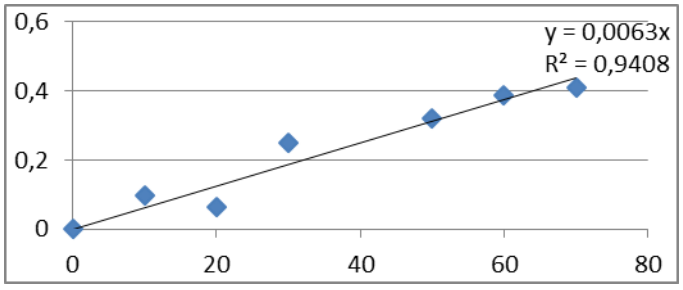

Fig. 8: Standard graph of protein estimation

CONCLUSION

From different fermented food such as rice, wheat, gram etc, total 3 isolates were heat tolerant, were primarily considered to be Bacillus species.

- All isolates are catalase positive.
- Out of these 3 cultures only 2 have protease activity.
- Preliminary test, such as gram staining shows these isolates were gram positive.
- Casein hydrolysis shows the selected isolates have protease activity.
- Among 3 isolates one shows Starch hydrolysis test positive and other two shows negative result.
- When selected isolates were inoculated in sugar sample- purple colour change into yellow which indicate utilization of sugar.
- Detection of Enzyme activity of the selected isolates was checked by Fibrinolytic plate assay, it shows clear zone on fibrin plate.

From the above work, it can be concluded that fibrinolytic enzymes produced from the Bacillus species that were isolated from fermented foods, has ability to degrade fibrin and isolates based on biochemical tests were identified to be (*Bacillus subtilis*), (*Bacillus fastidious*).

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