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RESEARCH ARTICLE

ISOLATION, IN VITRO CLOT LYSIS STUDY AND MOLECULAR IDENTIFICATION OF BACILLUS SP. ISOLATED FROM DIFFERENT SOURCES

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Abstract

Bacillus sp. possesses strong fibrinolytic properties. Out of 25 samples collected from different soil and water sources, 25 isolates were selected on the basis of colonial characteristics and Gram staining as Bacillus sp. Out of 25 isolates, 4 were selected based on the result of β- hemolysis and proteolytic activity. The result of invitro clot lysis and anti-thrombolytic assay revealed that N16 isolates showed highest activity (85%) and strong lysis with 100µl and 150µl of crude enzymes as compared to other 4 isolates. The molecular identification done by 16s r RNA sequencing of the selected 5 isolates showed 100% similarity with N-10 with Bacillus wiedmannii, N-14 with Bacillus safensis, N-15 with Bacillus inaqusorum, N-16 with Bacillus paramycoides. The study helps to find the role of fibrinolyticenzymes produced by Bacillus sp. with highest potential and to overcome the undesirable effects of commercially available thrombolytic agents.

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Introduction:-

Cardiovascular diseases such as myocardial infarction, strokes, high blood pressure, coronary heart diseases etc. have caused increased the death rate globally. One of the reasons for CVDs is thrombosis. Thrombosis occurs during the unbalanced state resulting in prevention of clot lysis. Fibrionolytic enzymes are used as therapeutic agents to treat thrombosis. They convert fibrin clots formed from fibrinogen by thrombin. Several fibrinolytic enzymes such as Staphylokinase, Nattokinase, and Streptokinase obtained from different organisms such as Staphylococcus sp., Bacillus sp., Streptococcussp. etc. respectively (Kim et al., 2000).Microbial fibrinolytic enzymes have grabbed the attention of researchers in past decade. Researchers are nowadays isolating this enzymes from variety of environmental samples to make it production cost effective and strategic planning is done to reduce its undesirable effects such as bleeding, unstability, low efficacy etc (Motaalet al., 2015).A huge number of fibrinolytic enzymes have been identified from various sources such as bacteria, fungi, and algae. Among bacteria, the members of the genus Bacillus have been extensively studied for fibrinolytic enzymes production and for their properties. Figure 1, indicates the action of Bacillus enzymes on fibrin clots. Orally administrable thrombolytic agents have drawn the attention of researchers which includes functional foods or drugs and its beneficial effects. The presence of fibrinolytic enzymes in food is a functional attribute. This has led to exploration for the similar and better kind of fibrinolytic enzyme extensively from various food sources (Yogeshet al., 2017).The present study focus on obtaining fibrinolytic enzymes produced by Bacillus sp. from different water and soil samples and studying the potent producer by in vitro clot lysis and anti-thrombolytic assay.

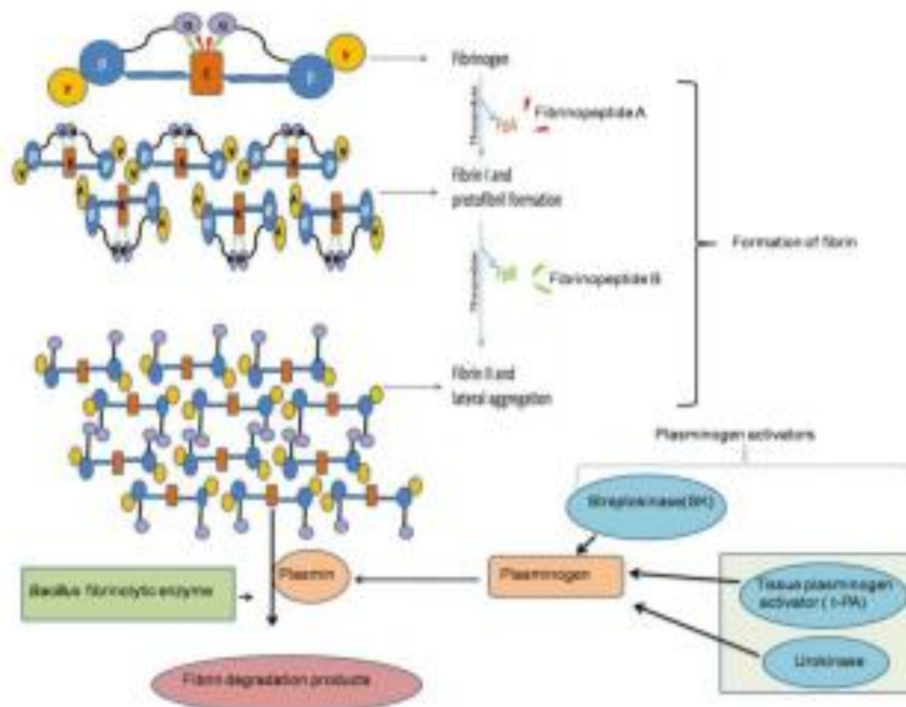

Fig 1:- Action of Fibrinolytic Enzymes produced by Bacillus Sp. (Yogeshet al., 2017).

Materials and Methods:-

Sample Collection:

25 samples of soil and water were collected fromSaputara, fish market, chicken market, soya sauce, vinegar and oyster sauce was collected from chinese stall and home. All the samples were collected in a sterile container and were stored in refrigerator till further use.

Isolation:

1 gm of soil sample was added to 9ml of normal saline, while water sample was taken in a sterile test tube and were kept in boiling water bath to kill the vegetative cells and other organisms present in the sample. Then one loopful of culture was streaked onto sterile nutrient agar plates and kept incubation for 24hrs at 37°C. Colonies were on basis of colonial characteristics appearing medium/large, round, dry, flat, irregular, opaque, creamish/white color colony was selected and supposed to be colony of Bacillus sp. The colonies were further screened on the basis of Gram's staining and morphological characteristicsas Gram positive long rods occurring singly or in chain. The selected isolates were purified and preserved on nutrient agar slant at 4°C for further use.

β-Haemolytic Activity:

Selected isolates were screened on blood agar plate for studying β-haemolytic activity by spot inoclutaion. The plates were then incubated at 37°C for 24 hours and next day zone of blood lysis was observed and measured by zone reader.

Proteolytic Activity:

Proteolytic activity was observed by doing spot inoculation of selected isolates on gelatin agar plates. The plates were then incubated at 37°C for 24 hours and next day zone of gelatin hydrolysis was observed and measured by zone reader.

Production Medium:

The isolates showing highest haemolytic and proteolytic activity was grown in production medium containing(gm/L) soyapeptone(10), K_2HPO_4(2), $MgSO_4$(1), maltose(20), yeast extract(10), glucose(29), pH 7.2 at 37°C for 24 hours.

Crude Enzyme Preparation:
After 24hours, 10ml of sample from the production medium was withdrawn aseptically in sterile centrifuge tube and was centrifuged at 2000 rpm for 20min at 4 °C. The supernatant was considered as crude enzyme and was further used to study invitro clot lysis and anti-thrombolytic assay.

Invitro Clot Lysis:
The weight of empty appendrof tubes was taken (W1). 1ml of blood withdrawn from healthy volunteer was transferred on to an appendrof tubes and the blood was allowed to clot at 37°C for 1 hour. After 1 hour, serum was removed and again the weight of appendrof tubes with blood clot was measured (W2). Then 1ml of crude enzyme was added to the appendrof tubes and incubated at 37°C for 90 min. After incubation the lysed part was discarded and the weight of blood clot measured (W3). For control, 1ml of blood was taken into an appendrof, allowed to clot at 37°C for 1 hour, serum was removed and 1ml of sterile production broth was added and incubated at 37°C for 90 min. The percentage of clot lysis was calculated for control tubes as well as tubes with crude enzyme by using the formula:

% of Clot Lysis: 100-{(W3-W1) / W2-W1)} X 100

Anti-thrombotic Assay: Whole blood was collected from healthy volunteer. 100µl of whole blood was taken in an appendrof tubesand 50µl, 100µl and 150µl of crude enzyme was added. The tubes were incubated at 37°C for 90min. After incubation the consistency of the blood was noted as weak, moderate and strong anti-thrombolytic activity.
Molecular identification: Molecular identification was done by 16s rRNA sequencing and the samples were sent to Saffron Life Sciences, Udhna-Surat.

Results and Discussion:-

Sample Collection:
25 samples were collected from different sources as shown in Table 1.and isolates were obtained.

Table 1:- Site and source of Sample Collection.

Sr. No.	Sample
1	OYSTERSAUCEAUCE
2	SOYA SAUCE
3	VINEGAR
4	FISH MARKET- WATER
5	CHICKEN MARKET- WATER
6	SOYA SAUCE
7	FISH MARKET- WATER
8	CHICKEN MARKET- WATER
9	FISH MARKET- WATER
10	CHICKEN MARKET- WATER
11	ANIMAL WASTE DUMP- SOIL
12	CHICKEN MARKET- WATER
13	CHICKEN MARKET- SOIL
14	FISH MARKET- SOIL
15	CHCHICKEN MARKET- SOIL
16	FISH MARKET- SOIL
17	FISH MARKET- SOIL
18	SOYA SAUCE
19	SOYA SAUCE
20	SOYA SAUCE
21	SOYA SAUCE
22	SOYA SAUCE
23	SOYA SAUCE
24	SOIL FROM SAPUTARA
25	WATER FROM SAPUTARA

Isolation:
25 isolates were selected on the basis on colonial, morphological characteristics and Gram's staining as shown in fig 2 and table 2.

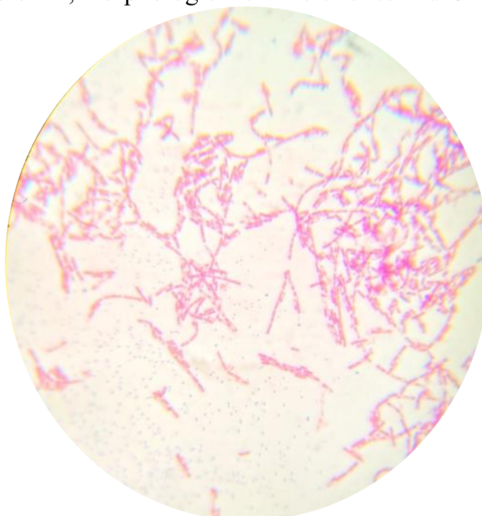

Fig 2:- Gram Staining of Selected Isolate.

Table 2:- Colonial and Morphological Characterisitics of Selected Isolates.

Colonial Characteristics	Large, Round, Dry, Irregular, Flat, Opaque, Creamish/White
Gram's Reaction and Morphology	Gram positive long rods occurring singly or in chain

β-Haemolytic assay:
Out of 25 isolates, 8 isolates showed haemolytic activity and among theses, N-10, N-14, N-15 and N-16 showed the highest activity as shown in fig 3 and table 3.

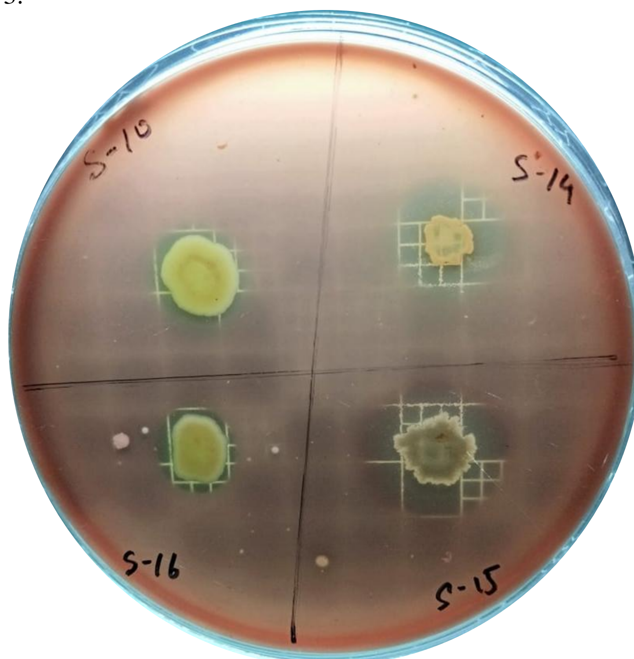

Fig 3:- β-Haemolytic Activity of Selected Isolates on Blood Agar Plates.

Table 3:- Result of Haemolytic Activity of Selected Isolates.

SamplSample Code	Result-β-Haemolysis(in mm) (in mm)
N8	11
N9	12
N10	15
N14	18
N15	19
N16	15
N17	11
N25	16

Proteolytic Assay:
All the 8 isolates obtained showed proteolytic activity on gelatin agar plate. N10, N14, N15 and N-16 showed the highest proteolytic activity as shown in fig 4 and table 4.

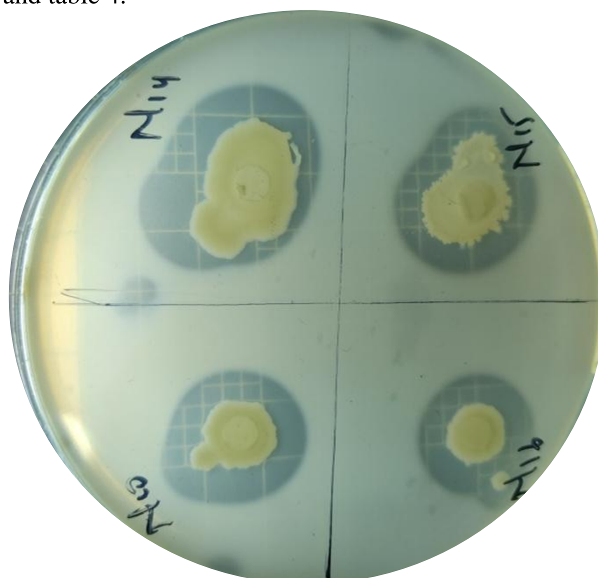

Fig 4:- Proteolytic Activity of Selected Isolates on Gelatin Agar Plates.

Table No. 4:- Result of Proteolytic Activity of Selected Isolates.

Sample Code	Proteolytic Activity (in mm)
N-8	12
N-9	11
N-10	24
N-14	27
N-15	22
N-16	20
N-17	18
N-25	10

Based on the result of β-haemolytic and proteolytic activity, N-10, N-14, N-15, N-16 was used for the further studies.

In Vitro Clot Lysis: The result of in vitro clot lysis revealed that N-10, N-14, N-15, N-16 showed 62%, 52%, 82% and 85% of clot lysis respectively as compared to control which did not show any lysis, as shown in fig 5 and table 5.

Fig 5:- In Vitro Clot Lysis of Selected Isolates.

Table No. 5:- Result of In Vitro Clot Lysis of Selected Isolates.

Sample Code	% of Clot Lysis
N-10	62
N-14	52
N-15	82
N-16	85

Anti-thrombotic assay:

The result of anti-thrombotic assay showed that N-10, N-14, N-15, and N-16 showed strong anti-thrombolytic affect with 100µl of crude enzyme as compared to control as shown in fig 6 and table 4. The consistency of blood showed that the addition of enzymes did not allow the blood to clot reflecting its anti-thrombotic property

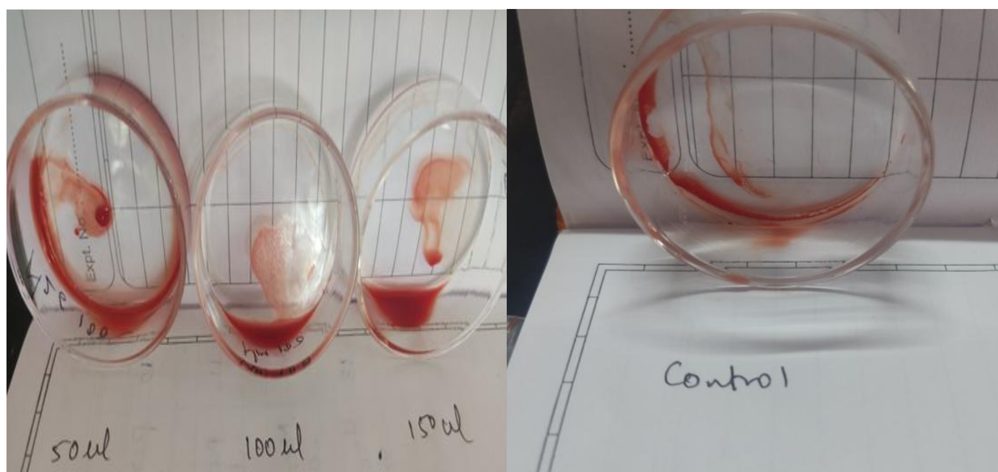

Fig 6:- Anti-thrombotic Assay of Selected Isolates.

Table No. 6:- Result of Anti-Thrombotic Assay of Selected Isolates.

Sample Code	Anti-thrombotic Assay			
	C	50µl	100µl	150µl
N-10	-	+	+++	++
N-14	-	+	+++	+
N-15	-	+	+++	++
N-1	-	+	+++	++

Molecular Identification:

The result of 16-s rRNA done in Saffron Life Sciences, Surat revealed that the selected four isolates were N-10 as Bacillus wiedmanni, N-14 as Bacillus safensis, N-15 as Bacillus inaquosorum and N-16 as Bacillus paramycoides as shown in fig 7.

Microbial Identification Report **SLS Research Pvt. Ltd.**

Protocol for Microbial Identification using 16s rRNA Gene

Experimental Method

1. DNA was isolated from the culture provided by the scientist. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed.

2. Fragment of gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose Gel.

3. The PCR amplicon was purified by column purification to remove contaminants.

4. DNA sequencing reaction of PCR amplicon was carried out with primer **1492R** using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.(Primer Details Given Below)

5. The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs.

Data and Result:

1. Sample which was labelled as **N10** showed similarity with ***Bacillus wiedmannii*** based on nucleotide homology and phylogenetic analysis.

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Sequences producing significant alignments:

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA, partial sequence	Bacillus wiedmannii	1903	1903	100%	0	100%	1540	NR_152692.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	Bacillus cereus	1903	1903	100%	0	100%	1486	NR_115526.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA, partial sequence	Bacillus thuringiensis	1903	1903	100%	0	100%	1486	NR_043403.1
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA, partial sequence	Bacillus toyonensis	1903	1903	100%	0	100%	1544	NR_121761.1
Bacillus paramobilis strain BML-BC017 16S ribosomal RNA, partial sequence	Bacillus paramobilis	1903	1903	100%	0	100%	1503	NR_175556.1
Bacillus sanguinis strain BML-BC004 16S ribosomal RNA, partial sequence	Bacillus sanguinis	1903	1903	100%	0	100%	1555	NR_175555.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA, partial sequence	Bacillus cereus	1903	1903	100%	0	100%	1535	NR_115714.1
Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA, partial sequence	Bacillus proteolyticus	1903	1903	100%	0	100%	1509	NR_157735.1
Bacillus pacificus strain MCCC 1A06182 16S ribosomal RNA, partial sequence	Bacillus pacificus	1903	1903	100%	0	100%	1509	NR_157733.1
Bacillus mobilis strain MCCC 1A05942 16S ribosomal RNA, partial sequence	Bacillus mobilis	1903	1903	100%	0	100%	1509	NR_157731.1

Microbial Identification Report **SLS Research Pvt. L**

Protocol for Microbial Identification using 16s rRNA Gene

Experimental Method

1. DNA was isolated from the culture provided by the scientist. Its quality was evaluated on 1.0% Agaros Gel, a single band of high-molecular weight DNA has been observed.

2. Fragment of gene was amplified by PCR. A single discrete PCR amplicon band was observed wher resolved on Agarose Gel.

3. The PCR amplicon was purified by column purification to remove contaminants.

4. DNA sequencing reaction of PCR amplicon was carried out with primer **1492R** using BDT v3.1 Cycl sequencing kit on ABI 3730xl Genetic Analyzer.(Primer Details Given Below)

5. The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Basec on maximum identity score first ten sequences were selected and aligned using multiple alignmen software programs.

Data and Result:

1. Sample which was labelled as **N14** showed similarity with ***Bacillus safensis*** based on nucleotide homology and phylogenetic analysis.

Microbial Identification Report **SLS Research Pvt. Ltd.**

Sequences producing significant alignments:

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus safensis FO-36b 16S ribosomal RNA, partial sequence	Bacillus safensis FO-36b	1768	1768	100%	0	99.90%	1434	NR_041794.1
Bacillus safensis strain NBRC 100820 16S ribosomal RNA, partial sequence	Bacillus safensis	1768	1768	100%	0	99.90%	1474	NR_113945.1
Bacillus australimaris strain MCCC 1A05787 16S ribosomal RNA, partial sequence	Bacillus australimaris	1757	1757	100%	0	99.69%	1513	NR_148787.1
Bacillus pumilus strain ATCC 7061 16S ribosomal RNA, partial sequence	Bacillus pumilus	1757	1757	100%	0	99.69%	1434	NR_043242.1
Bacillus pumilus strain NBRC 12092 16S ribosomal RNA, partial sequence	Bacillus pumilus	1757	1757	100%	0	99.69%	1474	NR_112637.1
Bacillus zhangzhouensis strain MCCC 1A08372 16S ribosomal RNA, partial sequence	Bacillus zhangzhouensis	1751	1751	100%	0	99.58%	1513	NR_148786.1
Bacillus aerius strain 24K 16S ribosomal RNA, partial sequence	Bacillus aerius	1746	1746	100%	0	99.48%	1466	NR_118439.1
Bacillus altitudinis 41KF2b 16S ribosomal RNA, partial sequence	Bacillus altitudinis 41KF2b	1746	1746	100%	0	99.48%	1506	NR_042337.1
Bacillus aerophilus strain 28K 16S ribosomal RNA, partial sequence	Bacillus aerophilus	1746	1746	100%	0	99.48%	1531	NR_042339.1
Bacillus stratosphericus strain 41KF2a 16S ribosomal RNA, partial sequence	Bacillus stratosphericus	1746	1746	100%	0	99.48%	1531	NR_042336.1

Microbial Identification Report SLS Research Pvt. Ltd.

Protocol for Microbial Identification using 16s rRNA Gene

Experimental Method

1. DNA was isolated from the culture provided by the scientist. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed.

2. Fragment of gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose Gel.

3. The PCR amplicon was purified by column purification to remove contaminants.

4. DNA sequencing reaction of PCR amplicon was carried out with primer **1492R** using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.(Primer Details Given Below)

5. The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs.

Data and Result:

1. Sample which was labelled as **N15** showed similarity with ***Bacillus inaquosorum*** based on nucleotide homology and phylogenetic analysis.

Microbial Identification Report SLS Research Pvt. Ltd.

Sequences producing significant alignments:

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus inaquosorum strain BGSC 3A28 16S ribosomal RNA, partial sequence	Bacillus inaquosorum	1977	1977	100%	0	100.00%	1538	NR_104873.1
Bacillus subtilis strain JCM 1465 16S ribosomal RNA, partial sequence	Bacillus subtilis	1977	1977	100%	0	100.00%	1472	NR_113265.1
Bacillus spizizenii strain NBRC 101239 16S ribosomal RNA, partial sequence	Bacillus spizizenii	1977	1977	100%	0	100.00%	1475	NR_112686.1
Bacillus subtilis strain NBRC 13719 16S ribosomal RNA, partial sequence	Bacillus subtilis	1977	1977	100%	0	100.00%	1475	NR_112629.1
Bacillus subtilis strain DSM 10 16S ribosomal RNA, partial sequence	Bacillus subtilis	1977	1977	100%	0	100.00%	1517	NR_027552.1
Bacillus spizizenii strain NRRL B-23049 16S ribosomal RNA, partial sequence	Bacillus spizizenii	1977	1977	100%	0	100.00%	1409	NR_024931.1
Bacillus tequilensis strain 10b 16S ribosomal RNA, partial sequence	Bacillus tequilensis	1977	1977	100%	0	100.00%	1456	NR_104919.1
Bacillus subtilis subsp. subtilis strain 168 16S ribosomal RNA, complete sequence	Bacillus subtilis subsp. subtilis	1971	1971	100%	0	99.91%	1550	NR_102783.2
Bacillus subtilis strain IAM 12118 16S ribosomal RNA, complete sequence	Bacillus subtilis	1971	1971	100%	0	99.91%	1550	NR_112116.2
Bacillus halotolerans strain LMG 22477 16S ribosomal RNA, partial sequence	Bacillus halotolerans	1971	1971	100%	0	99.91%	1468	NR_115931.1

Microbial Identification Report SLS Research Pvt.

Protocol for Microbial Identification using 16s rRNA Gene

Experimental Method

1. DNA was isolated from the culture provided by the scientist. Its quality was evaluated on 1.0% Agaro Gel, a single band of high-molecular weight DNA has been observed.

2. Fragment of gene was amplified by PCR. A single discrete PCR amplicon band was observed wh resolved on Agarose Gel.

3. The PCR amplicon was purified by column purification to remove contaminants.

4. DNA sequencing reaction of PCR amplicon was carried out with primer **1492R** using BDT v3.1 Cyc sequencing kit on ABI 3730xl Genetic Analyzer.(Primer Details Given Below)

5. The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Bas on maximum identity score first ten sequences were selected and aligned using multiple alignme software programs.

Data and Result:

1. Sample which was labelled as **N16** showed similarity with ***Bacillus paramycoides*** based on nucleotide homology and phylogenetic analysis.

Microbial Identification Report SLS Research Pvt. Ltd.

Sequences producing significant alignments:

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	Bacillus paramycoides	736	736	99%	0	91.01%	1509	NR_157734.1
Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, partial sequence	Bacillus tropicus	730	730	99%	0	90.83%	1509	NR_157736.1
Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA, partial sequence	Bacillus nitratireducens	730	730	99%	0	90.83%	1509	NR_157732.1
Bacillus luti strain MCCC 1A00359 16S ribosomal RNA, partial sequence	Bacillus luti	730	730	99%	0	90.83%	1509	NR_157730.1
Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial sequence	Bacillus albus	730	730	99%	0	90.83%	1509	NR_157729.1
Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA, partial sequence	Bacillus wiedmannii	725	725	99%	0	90.64%	1540	NR_152692.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	Bacillus cereus	725	725	99%	0	90.64%	1486	NR_115526.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA, partial sequence	Bacillus thuringiensis	725	725	99%	0	90.64%	1486	NR_043403.1
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA, partial sequence	Bacillus toyonensis	725	725	99%	0	90.64%	1544	NR_121761.1
Bacillus paramobilis strain BML-BC017 16S ribosomal RNA, partial sequence	Bacillus paramobilis	725	725	99%	0	90.64%	1503	NR_175556.1

Fig 7:- Molecular Identification of Selected Isolates by 16s rRNA.

Conclusion:-

From the present study it can be concluded that the four isolates identified as Bacillus spp. obtained from different sources possess strong fibrinolytic activity. These organisms can be further used for the production of thrombolytic agents which can act as a clot buster and the production cost can be decreased by using cheap substrate. The fibrinolytic enzymes obtained can be used as a food supplement and further studies can be done to assay its application as food additives and the advantage of it over the other commericially available thrombolytic agents.

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Conflict of Interest
No conflict of Interest is declared here.

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