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Screening of *Citrobacter* sp. for fibrinolytic protease production isolated from River Narmada

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Abstract
Prevention and therapy of thrombotic diseases have attracted much attention in developed countries during recent years. Lysis of preformed fibrin could be accomplished *in vivo* by plasmin enzyme led to an alternative enzyme-based approach. The drugs used for this therapy are called the fibrinolytic enzymes. In this study, w***e*** aimed to screen the ability of *Citrobacter* sp. (125 sp.) for Fibrinolytic protease production**.** Primary and secondary screening process was utilized to select the *Citrobacter* sp. which give the highest production of fibrinolytic enzyme and was found that *Citrobacter braakii* (BGCC#2123) had the highest productivity of the enzyme (35 FU/mg) and was designated as CbFP. The maximum activity of the enzyme was observed at temperature 30 ^{0}C and pH 8.0. Along with fibrinolytic activity *Citrobacter braakii* (BGCC#2123***)*** also posses gelatinase activity. *Citrobacter braakii* (BGCC#2123***)*** may be considered as a new source for thrombolytic agents.

Keywords: cardiovascular diseases fibrinolytic protease, *Citrobacter braakii*

1. Introduction

Fibrin is the main component of the blood clot, and is normally formed from fibrinogen by the action of thrombin (EC. 3. 4. 21. 5). The accumulation of fibrin in the blood vessels usually results in thrombosis. Thrombosis is one of the major cause of cardiovascular diseases (CVds). Cardiovascular diseases, including acute myocardial infarction, high blood pressure, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, stroke, etc. are the leading cause of death throughout the world and increasing rapidly in the developing countries.

Earlier day's treatment of cardiovascular diseases was relied on the use of anticoagulants, such as warfarin, coumarin and heparin to inhibit the formation of fibrin clots. Fibrinolytic drugs used for clinical applications are divided into three generations [1]. The first is composed of streptokinase (EC 3.4.99.0) [2], urokinase (EC 3.4.21.73) [3], staphylokinase (EC 3.4.99.22) etc. The second generation consists of tissue plasminogen activator (t-PA) [4], single-chain urokinase-type plasminogen activator (scu-PA, or pro-urokinase, pro-UK) [3], etc. The third generation is novel agents derived from the first or the second generations of thrombolytic agents by modern molecular biological techniques (mutants of pro-UK and t-PA) [5, 6]. Despite widespread use, these fibrinolytic agents suffer important shortcomings including bleeding complications, short half-life, high cost, the risk of allergic reactions, limited fibrin specificity, reocclusion, bleeding complications, dissolve necessary blood clotting protein and factor which are essential for normal homeostasis and large therapeutic doses [6]. Hence, it triggers us to search the novel molecule (safer and less expensive) or refinement to the existing one for ideal drug molecule.

Microbial fibrinolytic protease considered as a potent fibrinolytic agent to treat CVDs [7]. Because the ability of producing fibrinolytic enzyme from different strain is different from each other, acquiring new fibrinolytic agents through screening of novel strains with the highest fibrinolytic activity is the main task. Member of the genus *Citrobacter* belonging to the family Enterobacteriaceae have not yet been reported to produce fibrinolytic enzyme. Hence, it is worthwhile to screen genus *Citrobacter* for fibrinolytic enzyme secretion

2. Materials and Methods

2.1 Chemicals, reagents and blood specimen

Chemicals and reagent: Human fibrinogen**,** plasmin and thrombin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Other chemicals and analytical reagents were purchased from Himedia, Mumbai, India.

Blood Specimen: Whole blood (5 ml) was drawn from healthy human volunteers (*n* = 10) without a history of oral contraceptive or anticoagulant therapy. The informed consent from all volunteers was obtained in written.

2.2 Bacterial Strains

125 *Citrobacter* strains (44 were *C. koseri*, 38 were *C. freundii*, 18 were *C. braakii*, 07 were *C. sedlakii*, 05 were *C. murlinae*, 05 were *C. werkmanii*, 04 were *C. rodentium*, 03 were *C. youngae* and 01 was *C. gillenii*) were procured from the Bacterial Germplasm Culture Collection, Rani Durgavati University, Jabalpur (M.P.), India**,** which was previously isolated from river Narmada. The strain was maintained on Luria-Bertani (LB) agar slants (pH 7.0) at 37°C for 24 h and then stored at 4°C. Stock culture was subcultured in fresh LB agar slant after every 30 days.

2.3 Squential screening for fibrinolytic protease production

2.3.1 Protease activity assay

C*itrobacter* sp. were screened for extracellular proteases production by following the method of Deshmukh [8]. *Citrobacter* cultures were point inoculated on 0.4% gelatin agar plates (peptone, 5 g/L; beef extract, 3 g/L; sodium chloride, 5 g/L; gelatin, 4g/L; agar, 20 g/L) and incubated at 37±2 °C for 24 hr. After the incubation period, mercuric chloride reagent (1%) was poured directly on the cultures and zone of clearance was observed.

2.3.2 Qualitative fibrinolytic protease assay

All protease producing C*itrobacter* sp. were cultured in nutrient broth medium (peptone, 5 g/L; beef extract, 3 g/L; sodium chloride, 5 g/L) and incubated at 37°C in a shaker (150 rpm) for 24 h. 5 ml of incubated broth from each flask was centrifuged at 10,000 rpm for 15 min at 4 °C to remove cells. The cell free culture supernatant was used as source of extracellular fibrinolytic enzyme.

2.3.2.1 Fibrin agar plate method

Fibrinolytic enzyme production by C*itrobacter* sp. was screened by following the fibrin plate method of Astrup and Mullertz [9] with minor modification. A fibrinogen solution (2 ml of 50 mg/ml Bovine fibrinogen in 0.02 M Tris-HCl buffer, pH 7.4) was mixed with 40 ml of agar solution (0.75% (w/v) in 0.02 M Tris-HCl buffer containing 0.15 M NaCl, pH 7.4) at 55 °C, and 8 ml of this solution was poured into a petri dish with 40 µl of thrombin (100 NIH/ml Bovine thrombin in 0.02 M Tris- HCl buffer, pH 7.4). The plate was allowed to stand for 1 hr at room temperature for fibrin to clot, was heated at 80 °C for 30 min to destroy other fibrinolytic factors and wells were made on plate using cork borer (6 mm). 60 µl of the crude enzyme extract was then placed onto each well and incubated at 37 °C for 12 hr. After 12 hr of incubation, plate was observed for zone of hydrolysis and plasmin was used as standard enzyme.

2.3.2.2 Blood agar plate method

A fresh whole human blood was mixed with 2 % agar (0.02 M Tris- HCl buffer, pH 7.4) and poured into petri plate. Plate was allowed to stand for 1 hr at room temperature for clotting of blood and then wells were made on plate using cork borer (6 mm). 60µl of crude enzyme was then placed in each well and incubated for 12 hr. After 12 hr of incubation, plate*s* were observed for zone of fibrin hydrolysis and plasmin was used as standard enzyme.

2.3.3 Quantitative fibrinolytic protease assay

Fibrinolytic activity was measured with a fibrin degradation assay developed by Japan Bio Science Laboratory and followed by Wang *et al.* [1] with slight modification. 0.4 ml of 0.72% fibrinogen was placed in a test tube with 0.1 ml of phosphate buffer (0.245 M, pH 7.0) and incubated at 37 ºC for 5 min. Then, 0.1 ml of a 20 U/ml thrombin solution was added to it. The solution was again incubated at 37 ºC for 10 min and 0.1 ml of enzyme was added, and incubation continued at 37 ºC for 60 min. This solution was mixed every after 20 min. After incubation, 0.7 ml of TCA (0.2 M) was added, mixed and incubated at 37 ºC for 20 min. The reaction mixture was centrifuged at 13,000 rpm for 10 min. Then, 1 ml of supernatant was collected and absorbance at 275 nm was measured. One fibrin degradation unit (FU) of enzyme was defined as a 0.01-per-minute increase in absorbance at 275 nm of the reaction solution.

2.4 Effect of pH and temperature on the fibrinolytic enzyme activity

The effect of pH and temperature on fibrinolytic activity of enzyme was determined by incubating enzyme at different pH (5.0-9.0) and temperature (30º C to 60 °C). For pH standard buffers (50 mM) used were sodium acetate buffer (pH 5.0-6.0), sodium phosphate buffer (pH 7.0-8.0) and glycine sodium hydroxide buffer (pH 9.0). The enzyme activity was measured as stated above and was expressed as percentage relative activity with respect to maximum activity, which was considered as 100%.

3. Results & Discussion

Fibrinolytic enzymes are mainly proteases. These catalyze total hydrolysis of proteins and specifically act on interior peptide bonds [10]. All living cells produce different types of proteases, but the majorities are produced by microorganisms. Many workers have reported that bacteria are high protease producers [11]. Therefore, the quest for new plasmin- like bacterial fibrinolytic enzymes for use in thrombolytic therapy has never been suspended.

3.1 Protease activity assay

Owing to this application, 125 gram negative C*itrobacter* strains were screened for their potential to degrade fibrin, based on two strategies. First, screening was based on proteolytic efficiency by gelatin degradation. Among 125 strains, 90 C*itrobacter* strains show proteolytic efficiency by digesting gelatin (Fig. 1). These strains are gelatinase producers as they were able to digest gelatin and Gelatinase are widely used not only in chemical and medical industries but also in food and basic biological science [12]. Nowadays gelatinase have received considerable attention as targets for drug development because of their potential role in connective tissue degradation associated with tumor metastasis [13, 14]. So these 90 Strains can be utilized in future for their gelatinolytic activity.

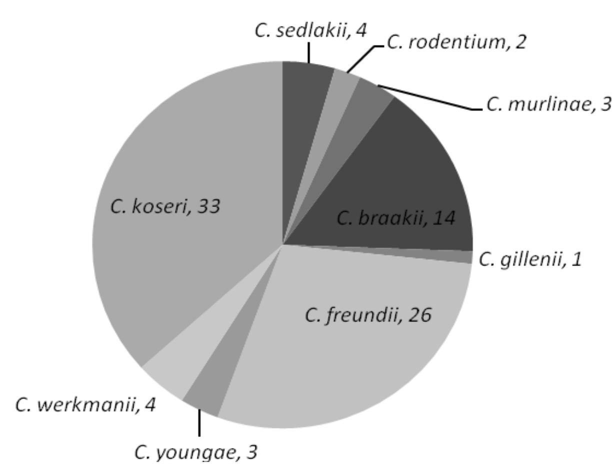
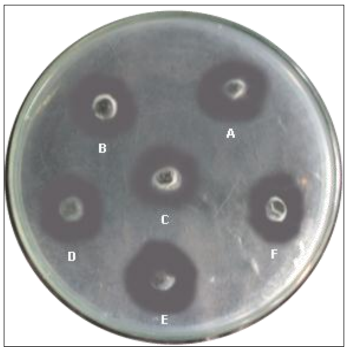


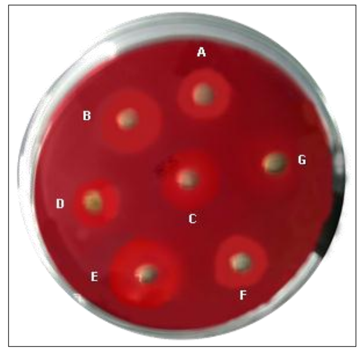
Fig 1: *Citrobacter* Sp. possessing protease activity

3.2 Fibrinolytic Protease activity assay

Since the aim of the present investigation was to search for potential fibrinolytic protease that would ultimately degrade fibrin, therefore in the second strategy extracellular fibrinolytic producers were screened. 90 protease-producing isolates were then screened for their fibrinolytic activity based on the zone of hydrolysis on fibrin agar and blood agar plates at 37°C. The zone of clearance was exhibited by only 20 strains due to the digestion of protein (fibrin) by the action of extracellular fibrinolytic protease among which *Citrobacter braakii* (BGCC#2123) showed significant fibrinolytic zone of 18.2 mm and 17.9 mm on fibrin and blood agar plate, respectively (Table 1, Fig. 2 a & b). *Citrobacter braakii* (BGCC#2123) possessed fibrinolytic activity of 35 FUmg^{-1} which was much higher as reported in *Pseudomonas* sp TKU015 after purification (28.9 Umg^{-1}) [1]. Gram-positive bacteria, particularly the genus *Bacillus* have known to be the prolific fibrinolytic enzymes producer[15]. However, only few extracellular fibrinolytic producer from gram negative bacteria viz. *Klebsiella aerogenes* and *Serratia marcescens* [16], *Pseudomonas* sp TKU015 [1] and *Bacillus* sp. strain AS-S20-I [17] have been reported. These results were suggestive of *Citrobacter braakii* (BGCC#2123) as potential isolate for production of extracellular fibrinolytic protease and enzyme was designated as *CbFP* (*Citrobacter braakii* fibrinolytic protease).



(a)



(b)

Fig 2: Degradation of fibrin protein by *Citrobacter* sp. on (a) Fibrin Agar Plate and (b) Blood Agar Plate where A: BGCC#2084, B: Plasmin (0.003 Units), C: BGCC#2092, D: BGCC#2170, E: BGCC#2123, F: BGCC#2177 and G: NaCl (0.85%)

Table 1: Screening of *Citrobacter* isolates for extracellular fibrinolytic enzyme production

S. No	Culture No.	*Citrobacter* isolates	Fibrinolytic activity		Fibrinolytic activity (FUmg^{-1})
			Fibrin agar plate (mm)	Blood agar plate (mm)	
1.	BGCC# 2062	*C. freundii*	7	6.2	20.0
2.	BGCC# 2075	*C. murlinae*	4.1	4.25	8.34
3.	BGCC# 2077	*C. murlinae*	5.4	5.56	12.12
4.	BGCC# 2081	*C. sedlakii*	3.7	3.85	5.88
5.	BGCC# 2084	*C. koseri*	15.2	14.8	30.0
6.	BGCC# 2089	*C. freundii*	13.8	13.5	25.0
7.	BGCC# 2092	*C. freundii*	6.7	6.8	10.0
8.	BGCC# 2099	*C. koseri*	13.20	13.6	25.12
9.	BGCC# 2106	*C. koseri*	7.2	7.0	13.57
10	BGCC# 2109	*C. koseri*	13.8	13.3	28.90
11.	BGCC# 2117	*C. koseri*	9.00	9.25	18.19
12	BGCC# 2122	*C. koseri*	14.00	13.7	29.12
13.	BGCC# 2123	*C. braakii*	18.2	17.9	35.0
14	BGCC# 2133	*C. freundii*	1.0	1.3	2.0
15.	BGCC# 2138	*C. freundii*	10.5	11.2	22.25
16	BGCC# 2147	*C. freundii*	15.8	16.0	31.50
17.	BGCC# 2156	*C. freundii*	12.2	8.9	28.0
18.	BGCC# 2168	*C. koseri*	3.2	3.0	10.91
19.	BGCC# 2170	*C. sedlakii*	15.5	4.2	28.36
20	BGCC# 2177	*C. werkmanii*	10.2	8.8	16.18

BGCC#: Bacterial Germplasm Culture Collection

3.3 Effects of pH and temperature on the fibrinolytic enzyme activity

The effect of pH on the crude *CbFP* activity was investigated at pH values ranging from 5.0-9.0 (fig. 3). The *CbFP* was found to be most active over a pH range 6.0-9.0 with maximum activity at pH 8 which is comparable to those of subtilisin FS33 (8.0) [13], lower than CDP (9.0) [19], subtilisin DFE (9.0) [20], but higher than those of MEF (7.0) [21], CMP-1 (7.0) [22], CSP (7.0) [23] and FVP-1 (6.0) [24], *Pseudomonas* sp. TKU015 (pH 7.0) [1].

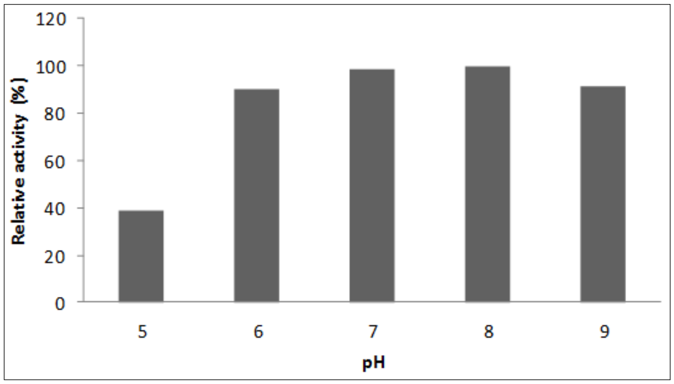


Fig 3: Effect of pH on activity crude CbFP

The optimum temperature for the crude *CbFP* activity was determined by measuring the relative enzyme activity at various temperatures ranging from 30-60 ºC. The *CbFP* was most active over a temperature range 30.0-50.0 ºC with maximum activity at 30 ºC (Fig 4) which is lower than that reported for AJ (85 ºC) [25], subtilisin FS33 (55 ºC) [18], subtilisin DFE (48 ºC) [20], N–V protease (45 ºC) [26], CSP (40 ºC) [23] but comparable to MEF (30 ºC) [21], *Bacillus* sp nov. SK006 [27] and *Flammulina velutipes* [24].

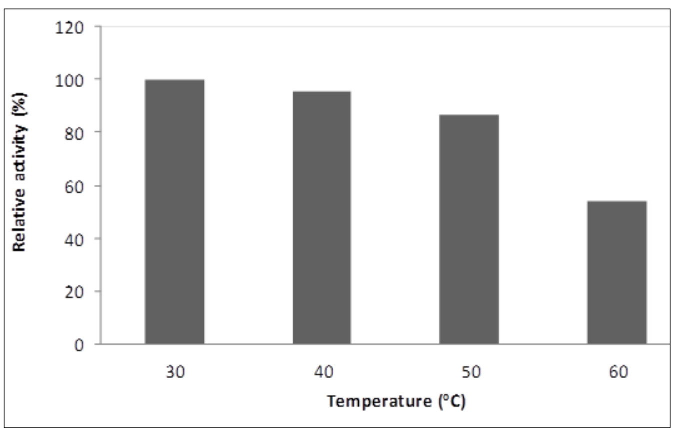


Fig 4: Effect of temperature on activity crude CbFP

4. Conclusions

In conclusion, fibrinolytic enzyme CbFP obtained from the *Citrobacter braakii* (BGCC#2123) exhibit profound fibrinolytic activity. *Citrobacter* sp. To the best of the knowledge there was no report on Genus *Citrobacter* possessing fibrinolytic activity. Gelatinase activity was also found among the members of this genus so for its industrial applications also this genus can be explored. Among genus *Citrobacter*, *Citrobacter braakii* (BGCC#2123) may become a new source for thrombolytic agents to treat thrombosis.

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