Biological Forum – An International Journal **15(3): 867-871(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Antiproliferative Effect of Fibrinolytic Enzyme and Apoptotic Gene Expression Studies on Human Carcinoma Cell Lines Produced from the Microorganism of Poultry Farm

Subha T.[1*], Sreeya G. Nair[2] and Beena Somanath[3]

[1]Research Scholar (Reg no: 20121172022030), Rani Anna Govt. College for Women, affiliated to Manonmaniam Sundaranar University, Tirunelveli /Assistant Professor, Department of Allied Health Sciences, NICHE, Kumarakoil (Tamil Nadu), India.

[2]Assistant Professor and Head of Zoology, Sree Ayyappa College for Women, Chunkankadai, Kanyakumari District (Tamil Nadu), India.

[3]Assistant Professor of Zoology, Rani Anna Govt. College for Women, Tirunelveli (Tamil Nadu), India.

(Corresponding author: Subha T.)*

(Received: 22 January 2023; Revised: 15 February 2023; Accepted: 28 February 2023; Published: 22 March 2023)
(Published by Research Trend)

ABSTRACT: Cancer is one of the serious disease, causes millions of human deaths worldwide and hence it is one of the most investigated research disciplines. The present study focuses on the antiproliferative effect and apoptotic gene expression of the fibrinolytic enzyme against cancer cell line. A newly isolated strain with fibrinolytic activity was screened from dumped soil enriched with poultry waste and the fibrinolytic enzyme produced in the optimised medium was purified. The final specific activity of the purified enzyme was found to be 1033.23 units/mg, which indicated that the enzyme was purified 21.08 fold with approximately 25% yield. Antiproliferative effect of the fibrinolytic enzyme on HeLa cell line revealed that the cell viability decreased with increased concentration of the enzyme. Apoptotic studies of fibrinolytivc enzyme showed that the gene expression of Bax was increased and Bcl_2 activity was decreased on cancer cell. The produced fibrinolytic enzyme showed its maximum anticancerous activity by decreasing the viability of cancer cells. In future this research area may result in the creation of using this novel fibrinolytic enzyme for the treatment of cancer.

Keywords: Poultry waste, Optimised medium, anti-proliferative effect, apoptotic studies, gene expression.

INTRODUCTION

Proteases are a class of enzymes which are very important for their application in physiological, Commercial, Pathophysiological and Pharmacological fields (Hariharan *et al.*, 2014). Fibrinolytic enzymes are the proteolytic enzymes involved in the degradation of fibrin clots (Krishnamurthy *et al*., 2018). Micro-organisms are the most important and cheap source of fibrinolytic enzyme (Collen and Lijinen 1994).

Soil Micro-organism has been investigated as potential bio factories for the synthesis of various enzymes. Enzymes produced by soil bacteria can provide numerous advantages over traditional enzymes owing to the wide range of environments from which they are recovered (Mander *et al*., 2011).

Both cancer and cardiovascular diseases are a serious threat to human health. Tumor growth and metastasis are closely related to the formation of new blood vessels and changes in the function of fibrinolytic system (Falanga *et al*., 2015; Hisano and Hla 2019). Currently fibrinolytic enzyme has been used as anticancer therapeutics including alkylating agents, antitumor antibiotics and antitumor drugs (Praveen *et al.*, 2019). Fibrinolytic enzymes and their activities displays antithrombotic effects including the inhibition of neovascularisation and hence prevention of cancer metastasis (Zhao *et al*., 2021). The aim of the present study is to produce the fibrinolytic enzyme and to analyse the anti-proliferative effect of crude enzyme isolated from the microorganisms of poultry farms.

MATERIALS AND METHODS

A. Sample collection

Soil samples were collected from various locations of poultry farm at Villukuri, Kanyakumari district. Samples were collected by scraping off the soil surface, and10 gm of soil were obtained from a depth of 25 cm.

B. Isolation of bacteria

Bacteria present in the soil was isolated by serial dilution and spread plated on Nutrient agar medium and 10^{-6} dilution was used for plating by spread plate method. The plates were incubated at 37˚C for24 hours. Pure bacterial isolates were obtained by repeated sub culturing on nutrient agar plates, and were stored in agar slants with the help of 15% glycerol.

C. Screening of fibrinolytic bacteria

Fibrinolytic activity was investigated by a fibrin plate assay as previously described (Astrup and Mullertz

1952), with minor modifications. The fibrin agarose gel (5-mm thick) contained 2.0% agarose, 0.12%(w/v) fibrinogen, 0.5 U/ml thrombin and 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl. The clot was allowed to set for 30 min at room temperature, after which cell-free bacterial culture supernatant of the selected proteolytic strain was carefully loaded onto the fibrin agar plate. Un-inoculated broth medium was used as a negative control. The loaded plates were incubated at 37°C for 24 hours, after which the clear zones around the paper discs indicating fibrinolytic activity were measured (Kumaran *et al*., 2011).

D. Production of fibrinolytic enzyme

Fibrinoytic enzyme production was carried out in a minimal medium composed of fibrin 2g (gm/l.), P^H - 8.8, Temp – 37°C, and 1% of inoculum was added. The Fermentation was carried out at 37°C for 14 hrs. After fermentation, the broth was centrifuged at 10,000 rpm at 4°C. The clear Supernatant was subjected to fibrinolytic activity (Chung *et al*., 2010). The Fibrinolytic assay was done under standard assay conditions (Smitha and Pradeep 2018).

E. Optimization of enzyme production

Production of fibrinolytic enzyme was optimized by supplementing the nutrient broth with inoculum, substrate fibrin, various carbon sources, organic nitrogen sources, inorganic nitrogen sources and natural substrates. Media Optimization has been successfully and efficiently applied for the production of numerous compounds and fermentation process (Venkataraman *et al*., 2010).

F. Purification process

The fibrinolytic enzyme was purified from the cell-free fermentation broth using a three-step purification process. The cell-free broth was subjected to ammonium sulphate precipitation (40% saturation) followed by dialysis and the dialysed enzyme solution was then passed through the gel filtration chromatography sephadex G100 column. The purification summary was calculated (Paik *et al*., 2004).

G. Determination of invitro antiproliferative effect of fibrinolytic enzyme on cultured HeLa cell line

HeLa cervical cancer cells were purchased from NCCS Pune and maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°C in 5 % CO_2 in a humidified atmosphere in a CO_2 incubator (NBS, EPPENDORF, GERMANY). The cells were trypsin zed (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution (HI media)) for 2 minutes and passaged to T flasks in complete aseptic conditions. 6.25 µl, 12.5µl, 25µl, 50µl and 100µl of sample was added to grown cells and incubated for 24 hours. The percentage difference in viability was determined by standard MTT assay after 24 hours of incubation (Mosmann, 1983).

The percentage viability was calculated using the following formula.

Percentage viability = (OD of Test/ OD of Control) × 100

H. Gene expression studies of Bax and Bcl2 on cancer cell lines

The SKMEL cell line was taken for the analytical studies. The cell lysate was prepared with Trizol reagent for the treated cell line along with the control. The RNA quality was checked by agarose gel electrophoresis and the quantification of RNA obtained was done using µDrop™ Duo Plate reader. cDNA was prepared using iScript cDNA synthesis Kit (Biorad).

The obtained cDNA was amplified using Bax, Bcl2 primers.

Primer	Primer Sequence
BAX F	AGGTTTGGGGCCACTATCTC
BAX R	GATCTGAAGATGGGGAGAGGG
BCl2 F	AACGCTTTGTCCAGAGGAGG
BCL2 R	GTACAGGGAAACGCACCTGA

The PCR reaction was carried out in 20µl reaction mixture containing 10µl Sso Advanced Universal SYBR Green supermix (2X, Biorad), 1µl of 10µM forward and reverse primer, 1µl of template DNA and 7µl Nuclease free water.

RESULTS AND DISCUSSION

1. Isolation of microorganism. The soil samples collected were serially diluted and 10^{-8} dilution was used for plating by streak plate method. The inoculated plates were incubated at 37°C in an incubator for 24 hours. Visible colonies were formed after incubation.

Fig. 1. Isolated colonies on nutrient agar plate.

2. Screening of microorganism: The potent fibrinolytic enzyme producing stain was screened on skim milk agar and fibrin plate. Our study colloborates with the results of Raju and Divakar (2013); Kapila *et al*. (2017).

3. Production of fibrinolytic enzyme: The production of enzyme significantly depends upon the media components and their interactive effect. Kapila Tanja *et al*. (2017) reported that the media composition is very important to determine their optimum level for maximum enzyme production. In this study the natural substrate cow dung provoked the enzyme activity. The media composition used is reported in Table 1.

Table 1: Composition of fibrinolytic enzyme producing medium.

Ingredients	Concentration w/100ml
Nutrient broth	1.3gm
Incubation period	16hrs
Temperature	42°C
Glucose	4
Sucrose	4
Fructose	4
yeast extract	0.6
soya bean	0.6
Beef extract	0.4
Fibrin	5g
Cow dung	6g
$MgSO_4$	0.02
$MgCl_2$	0.04
$MnSO_4$	0.03
$MnCl_2$	0.04
pH	8.8

4. Enzyme production in the optimized medium: The fibrinolytic activity was determined from control and optimized medium. The enzyme production high in the optimized medium was shown in Table 2. Optimisation of physical conditions such as Temp, pH, incubation time, carbon sources, nitrogen sources, metal ions and substrate showed high enzyme production. Similar results were also recorded by Gowthami and Madhuri (2021); Smitha and Pradeep (2018). Vijayaraghavan *et al.* (2016) reported in their study that the nutrient compositions, availability and cheap cost cow dung was used as the substrate for the production of fibrinolytic enzyme and the process parameters were optimised for the maximum production of the enzyme.

Table 2: Enzyme activity in the Optimized medium.

Particulars	Enzyme activity (U/mL)
Control medium	196.24
Optimized medium	672.34

5. Purification process. The Fibrinolytic enzymes have been successfully purified with a yield of 25%, purification fold 21.08 and specific activity 1033.23 U/mg. The Summary of purification was reported in Table 3.

Table 3: Summary of steps involved in purification.

Purification Steps	Total Protein (mg)	Total Activity (U/mL)	Specific Activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme	13.7	672.34	49.21	100	1
Ammonium sulphate precipitation	5.23	398.32	76.00	59	1.55
Dialysis	2.13	263.41	123	39.13	2.49
Sephadex G 100	0.16	169.32	1033.23	25.1	21.08

6. Determination of invitro antiproliferative effect of fibrinolytic enzyme on cultured HeLa cell lines

Table 4: Percentage viability of fibrinolytic enzyme.

Sample volume (µl)	Average OD at 540nm	Percentage Viability
6.25	0.1569	90.69364
12.5	0.1408	81.38728
25	0.1273	73.58382
50	0.1005	58.09249
100	0.0894	51.6763

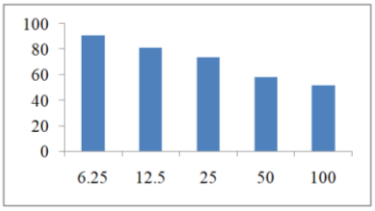

Fig. 2. Graph showing percentage viability.

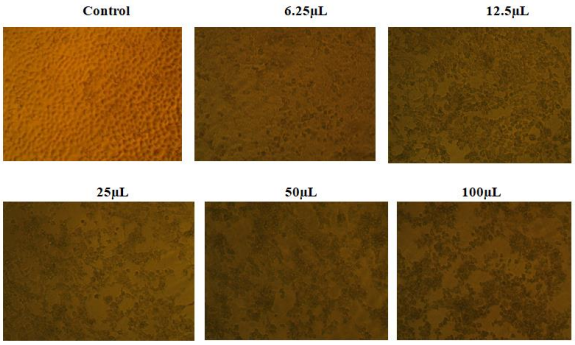

Fig. 3. Antiproliferative effect of fibrinolytic enzyme on cultured HeLa cell lines.

Table (4) illustrates the effect of fibrinolytic enzyme in destroying He La cell lines. Capability of cell survival was markedly lowered when compared to control. About 51.67% cell death was noticed at 100 µL of enzyme. The fibrinolytic enzyme inhibited the growth and migration of He La cell lines in a dose dependent manner (Fig. 2). The results revealed the therapeutic potential of the novel poultry house fibrinolytic enzyme and also it displayed an antitumor effect on He La cell lines in vitro. Similar results were observed by Liu *et al*. (2017) on Breast cancer cell line.

7. Gene expression studies of Bax and Bcl2 on cancer cell lines

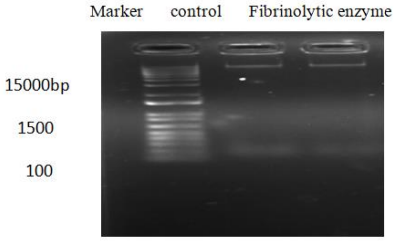

Fig. 4. Gel picture of isolated RNA

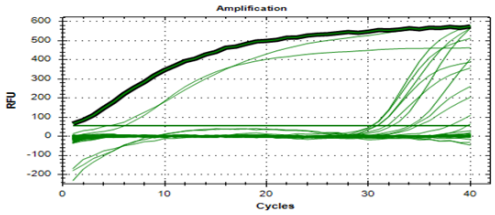

Fig. 5. log amplification curve of control and sample in Bax primer.

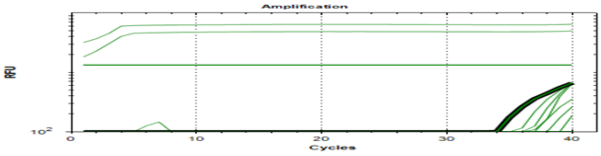

Fig. 6. Log amplification curve of control and sample in Bcl2 primer.

In the present study, RT-PCR analysis was done to check the expression of two apoptotic genes Bax and Bcl2 when it was treated with fibrinolytic enzyme . The mRNA expression of Bax was increased and the expression of Bcl2 was decreased. It indicates that fibrinolytic enzyme has significant effect on apoptosis as it increased the expression of Bax in SK-MEL cell line.

RTPCR data indicated that the fibrinolytic enzyme treatment induced the down regulation of Bcl2 genes and up regulation of Bax members. These findings suggest that the fibrinolytic enzyme is associated with the induction of apoptotic cell death. This study collaborates with Baik Hyun *et al*. (2013). Nachmias *et al*. (2004) suggested that the induction of apoptosis is to be an efficient strategy for the treatment of cancer.

CONCLUSIONS

Our data exhibited that the crude fibrinolytic enzyme purified from poultry farm has potential antiproliferative effect on cancer cell and also revealed significant expression of apoptotic genes on cancer cell.

FUTURE SCOPE

The purified enzyme obtained from this study has antiproliferative effect thereby it can be used as pharmaceutical agents. More future works are required for its in vivo usage process.

Acknowledgement. We the authors acknowledge the support extended by each and every one for the successful completion of this project work.

Conflict of Interest. None.

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How to cite this article: Subha T., Sreeya G. Nair and Beena Somanath (2023). Antiproliferative Effect of Fibrinolytic Enzyme and Apoptotic Gene Expression Studies on Human Carcinoma Cell Lines Produced from the Microorganism of Poultry Farm. *Biological Forum – An International Journal, 15*(3): 867-871.