Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Der Pharmacia Lettre, 2016, 8 (6):23-28
(http://scholarsresearchlibrary.com/archive.html)

Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Molecular confirmation and Role of carbon source for Biosynthesis of fibrinolytic enzyme by *Aspergillus tamarii*

Shilpa H. K.[1], Jeevan G. Ambekar[1], Basawaraj. B. Devaranavadagi[1], Nilima N. Dongre[1] and Siddalingeshwara K. G.[2]

[1]*Department of Biochemistry, Shri B.M. Patil Medical College, Vijayapura, Karnataka, India*
[2]*Scientific & Industrial Research Centre, Bangalore*

ABSTRACT

Fibrinolytic enzymes are agents that dissolves fibrin clots extracted from microbes were considered to be a potential thrombolytic agent for the treatment of clot-dissolving in the cases of Myocardial infarction. The fungal strains i.e Aspergillus tamari SAS 02 were isolated from different soils from different regions of Karnataka and screened for fibrinolytic activity by plate assay .The potential strain Aspergillus tamarii SAS 02 were used for the enhance the biosynthesis of fibrinolytic enzyme by supplementation of carbon sources were employed in range of 0.25% to 1.25%. The carbon source were supplemented are glucose, sucrose and maltose. Among the carbon source the glucose showed better yield of 186 IU fibrinolytic enzyme production and maltose and sucrose showed 109 IU and 86 IU.

Key words- fibrin, carbon source, *Aspergillus tamari* and Moleculat identification

---

INTRODUCTION

Thrombosis, that is the formation or presence of a thrombus (a clot of coagulated blood attached at the site of its formation), in a blood vessel is one of the most widely occurring diseases in modern life, which often causes disturbance in haemostasis a leading cause of cardiovascular diseases causing disability and death. According to world health organization (WHO), 17 million people die of such cardiovascular diseases (CVDs) every year [1].

Consequences like imbalance in the haemostasis i.e. balance between coagulation and anticoagulation, may result in excessive bleeding or formation of a thrombus that can adhere to the unbroken wall of the blood vessels. Fibrin can accumulate in the blood vessels which can interfere with blood flow and lead to myocardial infarction and other serious cardiovascular diseases. Unless the blockage is removed promptly, the tissue that is normally supplied with oxygen by the vessel will die or be severely damaged [2].

Fibrinolytic enzymes have been purified, cloned and studied from many plants, animals and microbial sources [3]. The agents are of interest as useful tools for understanding fibrinolytic mechanism and as potential therapeutic drugs.

Due to the importance of fibrinolytic enzymes production, we made an effort to synthesis of fibrinolytic enzyme from *Aspergillus tamari* through submerged fermentation, achieved an enhanced level production of fibrinolytic enzyme by supplementation carbon source were carried out.

MATERIALS AND METHODS

Microorganism

The *Aspergillus tamarii* strains were isolated from different soil samples. Soils are taken from different regions from in and around Bangalore and tentatively identified as per Refer and Fennel [4] in the laboratory and confirmed by molecular level identification.

Screening of fibrinolytic enzyme producers by plate assay

Aspergillus tamari were used to screen by fibrin plate assay [5]. A mixture consisting of 2 ml of fresh, healthy human plasma and 3 ml of 1.2% molten agarose (450C) in 10 mMTris-HCl buffer containing 70 mM (NH4)2 SO4, 90 mM NaCl, 0.70 mM MgCl2 and 200 µl of 0.2 M CaCl2 was poured into sterile 60 mm petridish and allowed to stand for 2 h at room temperature (25 to 27^0C). 10 ml of Sabouraud dextrose broth was inoculated with the given fungal strain and incubated at 30^0C for 2 days. This culture was used for fibrin clot assay. 20µl of fungal culture containing mycelia was placed at the center of the gel matrix of the fibrin plate and incubated for 24 h at 300C. The diameters of the clear zones (plaque) were noted.

Identification of fungal strain by molecular level

1. DNA extraction

The genomic DNA was isolated from the fungus using fungus genomic DNA extraction.

2. PCR Amplification:

Amplification of the ITS region (ITS1, ITS4 and 5.8 S rRNA Gene) for fungus sample was performed using following primer.

ITS Primers (5' to 3')

ITS1 F	TCCGTAGGTGAACCTGCGG
ITS4 R	TCCTCCGCTTATTGATATGC

PCR was performed as follows in a total volume of 50 µl in a 0.2 ml thin walled PCR tube.

Components	**Volume**
Nuclease free water	32 µl
Colony from plate	2.0 µl
Forward Primer (10µM)	2.0 µl
Reverse Primer (10µM)	2.0 µl
5x Reaction Buffer	10 µl
dNTP Mix (10 mM)	1 µl
Taq DNA polymerase (2.5U/µl)	1µl
Total volume	**50 µl**

The amplification was carried out in a Thermocycler (DNA-AMP Bhat Biotech) using the following program. Initial Denaturation was carried out at 94^oC for 10 minutes followed by 35 cycles of Denaturation at 94^oC for 1 minute, annealing at 56^oC for 1 minute and extension at 72^oC for 1 minute. Final extension was carried out at 72^oC for 10 minutes. The PCR products were purified to remove unincorporated dNTPS and Primers before sequencing using PCR purification kit (GENEASY GEL ELUTION KIT, Bhat Biotech India Pvt Ltd).

3. Sequencing:

Both strands of the rDNA region amplified by PCR were sequenced by automated DNA sequence -3037*xl* DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1cycle sequencing Kit (Applied Biosystems). Sequence data were aligned and dendrograms were generated using Sequence analysis software version 5.2 from applied biosystems. The sequences obtained for plus and minus strands were aligned using appropriate software before performing bioinformatics.

4. Bioinformatics analysis:
Sequences were compared to the non-redundant NCBI database using BLASTN, with the default settings used to find sequences closest to each other. The Expected value and e values were noted for the most similar sequences. Ten similar neighbors were aligned using CLUSTAL W2. The multiple-alignment file thus obtained was then used to create a Phylogram using the MEGA5 software.

Influence of carbon source for the biosynthesis of fibrinolytic enzyme
A set of conical flasks with 100 ml of production medium supplemented with a particular carbon source with concentrations ranging from 0.25% to 1.25% with increments of 0.25%. The different carbon sources like, monosaccharides (glucose) and disaccharides (Sucrose and Maltose) were used under the present study. The production medium consists (mg/100 ml) of Sucrose 3, di potassium hydrogen phosphate 0.1, MgSO4,0.05g, KCl 0.05g, NaCl, 0.01%, FeSO4 and devoid of sucrose. The condition of the fermentation medium is as fallows .pH,6 temperature 40^0C and inoculums size is of 1.25ml.

Extraction of fibrinolytic enzymes
The samples were withdrawn periodically at 24 hrs in aseptic condition. The extract was filtered through What'sman filter No.1. The clear extract was centrifuged at 2000-3000 rpm for 15 min, supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for assay of fibrinolytic enzyme.

Enzyme Assay
This was basically measured by the modified method of Anson [6], but with a few modifications. The reaction mixture contained 1 ml of 1.2% of bovine fibrin solution in Tris-HCl buffer (pH 8.0) and 1 ml of cell-free supernatant (CFS). The reaction mixture was incubated for 2 h at 37°C. Then the reaction was stopped by the addition of 2 ml of 10% (w/v) trichloroacetic acid. This was followed by centrifugation and assaying the solubilized proteins for tyrosine in the supernatant by measuring the absorbance at 750 nm [7]**.**

UNIT
One unit of fibrinolytic activity (U) was defined as the amount of enzyme required to liberate 1 µg of L-tyrosine/ml/min at 37°C

RESULTS AND DISCUSSION

Aspergillus tamari strain were isolated and screened for fibrinolytic enzyme production by plate assay and the strain were confirmed by molecular level identification. The potential strain were labeled as *Aspergillus tamari* SAS 02(Plate-1).

The Amplification of the ITS region (ITS1, ITS2, and 5.8 S rRNA Gene) were run on 1.0 % Agarose gel electrophoresis showed PCR product of ~600bp and represented plate-2. The aligned sequence of ITS region were represented in plate-3.

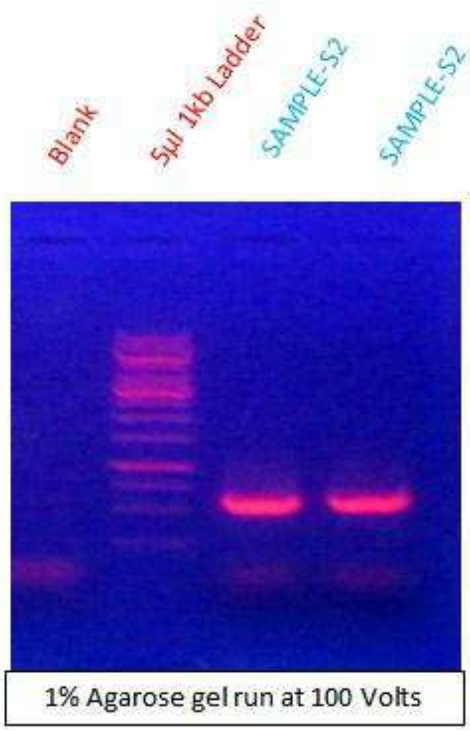


Plate 2: Individual Colonies were used for direct colony PCR and the product was run on 1% Agarose gel. The 600 bp amplicon were cut out from the gel, purified and sent for sequencing.

ITS1 F

TNGAGNGTAGGGTTCCTAGCGAGCCACCTCCCACCCGTGTTTACTGTACCTTTGTTGCTTCGGGGGGGCCGGCCTTTA
AGGCCGCCG
GGGGGGTCCAATCGGGAAATAGTTCCCTCCGTGGTTGTTTTTTTGAGGCCGGCGGCAACCCAGACCATCCTTTTATTA
AAATTTTTA
AACGGAAGCGATACAAATTCAAGTCGAATTCTANCAAATAAGATTTTGTTTTTTTTTCTGCCGCGGGCCGGCCTGATT
CACCCCCCC
CCGGCGCCCCTGGAGGGGCCGGCCCGCCGCAAGCAAATATACACAGACAACAGGGGGGGGGGGGGGGGCGGTAGACC
CCACACTTA
GTACTTCTTCCGTAGGACAACCTGAGGAAGCATCATTACCGAGTGTGGGGTTCGTAGGGCCCCCAACCTCCCACCGGT
TTTTATTAC
ACTTTAGTTGTTTGGGGGGGCCCGCCTTTCATGGCCGGGGGGGGTTATCACCCCGGGGCCCGCCCCCGCGGGAGACAA
CAAAAACTG TGTGATATATAGTGATGTGTGAGTTGATTG

ITS4 R

TATCACAATCAGTTAAAATTTTAAAAAATGGATCTTTGGGTTCGGGCATCGGAGAAAAACGCAGAAAAATGAGAAAA
ATAGTGAA
AATTGCAAAATTCCGAGAATCATAGAGTTTTTAAACACACATTGCGCCCCGGGGTATTCGGGGGGACACGCGTGTCAG
AGTCTCAT
TGGTGCCCAAAAAGCAGGGTTTGTGTGTTGTGTTGTTGTCCCCTTTCGGGGGGGGAGGGGCCCAAAAGGGAGGGGGG
GCAGCGTGT
CATCTCCTAGAGTATATGGGGTTTCTCACCCTCTGTATAGGCCGGGCGGGCGCTTGGAAAACAAAACAAAATTTTTCC
AGGTGACCT CGGATCAGTAGGANN

Plate-3: Aligned sequence of ITS region

The sequence of the ITS gene from sample-1 and that of matching sequences from 10 nucleotide sequences were

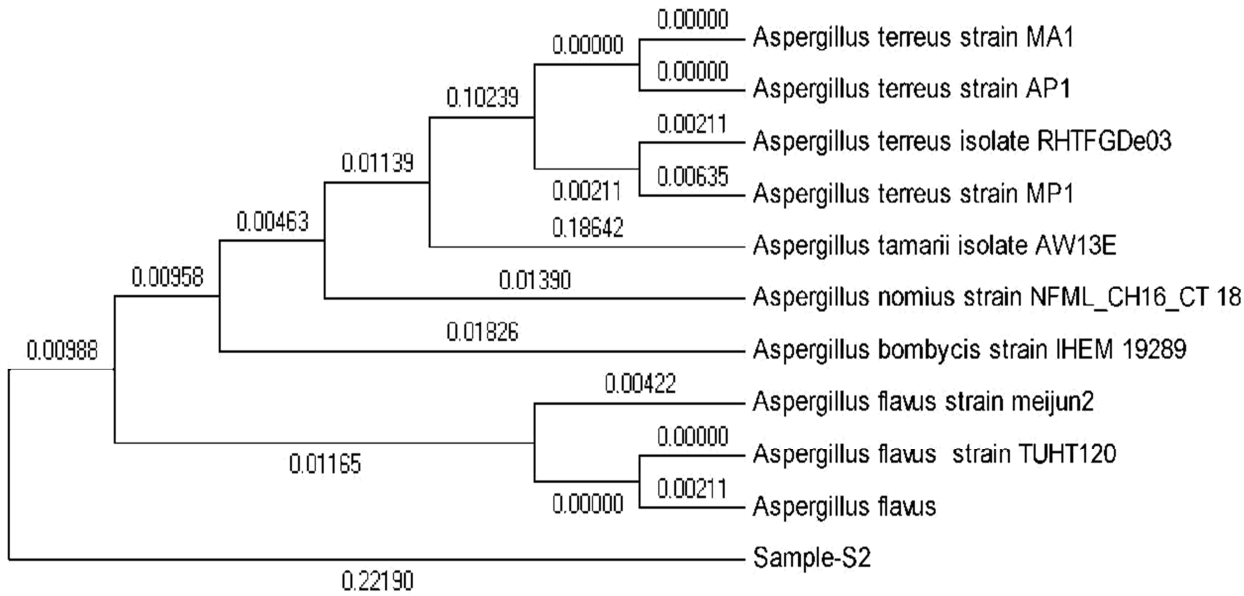
aligned by using the Maximum Likelihood method based on the Tamura-Nei model [8]. The tree with the highest log likelihood (-1749.6508) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 476 positions in the final dataset. Evolutionary analyses were conducted in MEGA5(Plate-4).

Plate-4: Phylogenetic tree

The process economization for fibrinolytic enzyme production with carbon sources supplemented to the production medium were carried out with concentration of 0.25%, 0.5%, 0.75%,1.0% and 1.25%. The results revealed that all the carbon sources employed under the present study have enhanced the production of fibrinolytic enzyme upto1.0% of monosaccharide and disaccharides (glucose, sucrose and maltose) at 72 hrs of fermentation represented in Fig1-3, thereafter no significant production of fibrinolytic enzyme was observed on all the days of fermentation period. In case of monosaccharides (glucose) the maximum fibrinolytic enzyme production of 186 IU was observed at 1.0% and where as disaccharides like maltose and sucrose yielded maximum fibrinolytic enzyme of 109 IU and 86 IU.

Essam et al., [9] were reported that six types of carbon sources were investigated: maltose, manitol, fructose, glucose, sucrose, lactose at glucose is a generally preferred carbon source for growth of bacteria and it was used as reference. The other five carbon sources had all been reported to be the best in respect of protease production by strains of *Bacillus lichniformis*. The results showed that, based on fibrinolytic production, mannitol was the optimal carbon source (46 unit/mL). Glucose, maltose and sucrose had similar positive effects, while lactose and sucrose performed poorly. So maltose was chosen as carbon source for the following investigations. Wang *et al.* [10] who showed that, based on nattokinase production, maltose was the optimal carbon source.

Rashmi and Liny [11] were reported that the carbon source, maltose showed the 2.4mg/ml of fibrinolytic enzymes in *Aspergillus niger* and 2.2mg/ml in *Aspergillus flavus*. Glucose showed high production rate in 2.2 mg/ml in *Penicillium notatum* which was relatively higher comparable to 64mg/l in *Bacillus sphaericus* (10). Our results were coincides with the results of Essam et al., [9].



CONCLUSION

The present work highlights on production of fibrinolytic enzyme from *Aspergillus tamarii* SAS 02 through submerged fermentation. The isolation and screening were carried out. The process economization were carried out

by supplementation of carbon source for enhanced level of fibrinolytic enzyme and it is achieved by supplementation of glucose at 1% level to the fermenting medium.

REFERENCES

[1] Y Peng, Y Xiao-Juan and Z Yi-Zheng, *Applied Microbiology & Biotechnology*. **2005,** 69: 126–132.
[2] Y Mine, H K W Ada and J Bo. *Food Research International*, **2005,** 38: 243–250.
[3] K Balaraman and G Prabakaran. *Indian Journal of Medical Research*, **2007**, 126: 459-464.
[4] KB Rapper and DL Fennel, The genus *Aspergillus*, Williams and Wilkins, New York. **1965,** pp. 567-577.
[5] T Astrup and S Mullertz, *Arch Biochem* **1952**, 40, 346-51
[6] M L Anson, J *Gen. Physiol*., **1939**, 22, 79–89.
[7] D J Mukesh Kumar, R Rakshitha, M. Annu, P.Vidhya. Sharon Jennifer, Sandip Prasad, M. RaviKumar and P.T. Kalaichelvan. P *Pakistan Journal of Biological sciences*, **2013**,17 (4), 529-234.
[8] K Tamura and M Nei. *Mol BiolE,* **1993,** 10, 512–526.
[9] F Essam. Al-Juamily and Bushra H Al-Zaidy. *British Journal of Pharmacology and Toxicology* 3(6): 289-295
[10] S Wang, H Chen, T Liang and Y Lin, *Process Biochem*, **2009**, 44: 70-76.
[11] Rashmi and Liny. *Int J Pharm Bio Sci*: **2013**, 4(3): (B) 454 – 463