



## *Stenotrophomonas maltophilia* Gd2: A potential and novel isolate for fibrinolytic enzyme production

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### ABSTRACT

The bacterium with an ability to produce extracellular fibrinolytic protease was isolated and identified as *Stenotrophomonas maltophilia* Gd2 based on ribotyping. The *in-vitro* fibrinolytic profile of this enzyme depicted 73% of fibrin clot dissolution within 4 h. Fibrinolytic enzyme yield influenced by different physiological (incubation time, temperature, agitation and pH), nutritional (macronutrients such as carbon and nitrogen sources) and biological (inoculums age and inoculums concentration) parameters of fermentation which were optimized based on one-factor-at-a-time (OFAT) approach. The enzyme yield improved from 886 to 1795 FU ml<sup>-1</sup> upon OFAT; optimized conditions include temperature – 33 °C, pH – 8.0, incubation time – 36 h, agitation – 150 RPM, 3% v/v inoculums and age of inoculum – 18 h. Further optimization of enzyme production was achieved with implementation of Plackett-Burman media designing where the production levels increased to 3411 FU ml<sup>-1</sup> and noticed that peptone, pH, dextrose and K<sub>2</sub>HPO<sub>4</sub> was found to be significant factor. This ms reports the highest fibrinolytic enzyme yield with *S. maltophilia* to that of literature reports.

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## 1. Introduction

The cardiovascular diseases (CVDs) have emerged as one of the devastating ailments in global health apparent through coronary heart disease like embolism, myocardial infarction, stroke and other cardiac disorders (Bajaj et al., 2014). WHO estimated, in 2015, that approximately 17.7 million people died from CVDs, representing 31% of all global deaths and the figure is predicted to rise to 25.6 million by 2020. Most of the CVDs related deaths (approximately 82%) occurred in third world and low-income countries (<http://www.who.int/mediacentre/factsheets/fs317/en/>).

Deep Vein Thrombosis (DVT), one of the major causes of CVDs, is associated with fibrin clot. In normal physiology of haemostasis there is balance between the formation and degradation of fibrin

however this is disturbed in pathophysiological disorders, leading to accumulation of fibrin which in turn causes DVT (Kotb, 2013). Synthetic anticoagulants (warfarin/coumarin and heparin) though inhibit the formation of fibrin clots, but not lyse the preformed clot hence have less significance in the DVT treatment (Balaraman and Prabakaran, 2007). Currently various fibrinolytic enzymes such as bacterial plasminogen activator, streptokinase, urokinase (u-PA) and tissue Plasminogen activator (t-PA) are being extensively investigated and widely used for treating the DVT (Turpie et al., 2002; Blann et al., 2002). These thrombolytic enzymes have edge over chemical anticoagulants due to substrate specificity (Kotb, 2013).

Microbial thrombolytic biocatalysts play significance in process economics to that from snake venoms (De-Simone et al., 2005), centipede venoms (Jia et al., 2003), insects (You et al., 2004) and earthworms (Wang et al., 2005). Fibrinolytic enzyme production by genus *Bacillus* from fermented foods such as natto (Fujita et al., 1993), Joetgal (fermented fish) (Kim et al., 1997), douchi (Peng et al., 2003) and doenjang (Choi et al., 2005) as well as streptokinase from *Streptococcus haemolyticus* (Billroth, 1874) and staphylokinase from *Staphylococcus aureus* (Gerheim, 1948) have been investigated most extensively. These fibrinolytic enzymes show indirect fibrinolytic activity i.e. they act like tissue

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Plasminogen activator which converts inactive plasminogen to active plasmin; an indigenous fibrinolytic enzyme cleaves pre-formed thrombus and restores the normal blood flow. Because of their indirect fibrinolytic activity they required to be given in high dose which caused various other side effects. To counter this problem researches have started to explore the fibrinolytic enzyme which directly act on fibrin clot. Recently [Bhargavi and Prakasham \(2016\)](#) reported serralysin from *Serratia marcescens* RSPB11 which revealed direct fibrinolytic activity ([Bhargavi and Prakasham, 2016](#)).

Bioprocess is one of the important components in improving the yield. Most of the studies reported on fibrinolytic enzyme include the isolation, purification and characterization of the enzyme, and a few reports are available in fibrinolytic enzyme related to optimization ([Bajaj et al., 2014](#); [Plackett and Burman, 1946](#); [Mahajan et al., 2012](#)). Process optimization is useful for screening large number of process variables, and to select variables that can be retained or eliminated to improve process economics ([Bhargavi et al., 2012](#)). The present ms is focused on development of bioprocess targeting yield improvement of fibrinolytic enzyme from isolated *Stenotrophomonas maltophilia* Gd2 using one-factor-at-a-time approach initially followed by Plackett-Burman media designing and authors report a four-fold improvement in enzyme production.

## 2. Material and methods

### 2.1. Chemicals and reagents

Starch, casein, agar, dextrose, peptone, nutrient broth, nutrient agar, yeast extract powder and dialysis membranes-120 were purchased from HiMedia laboratories, India. Thrombin, fibrinogen (human origin) and bovine serum albumin powder were purchased from Sigma-Aldrich, India. Trichloroacetic acid (TCA) was from purchased from Finar Chemical Ltd, India.  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ ,  $\text{K}_2\text{HPO}_4 \cdot 6\text{H}_2\text{O}$ ,  $\text{KH}_2\text{PO}_4 \cdot 6\text{H}_2\text{O}$  and other salts were purchased from Himedia, India. All other chemicals and reagents used were of analytical grade.

### 2.2. Isolation of fibrinolytic enzyme producing bacteria and activity evaluation

Bacterium producing fibrinolytic enzyme was isolated using soil collected from Godavari and Ganges rivers using casein agar plates containing peptone –  $5.0 \text{ g L}^{-1}$ , Yeast extract –  $1.0 \text{ g L}^{-1}$ , Beef extract –  $5.0 \text{ g L}^{-1}$ , NaCl –  $15.0 \text{ g L}^{-1}$ , Casein –  $30 \text{ g L}^{-1}$ ,  $\text{K}_2\text{HPO}_4$  –  $0.1 \text{ g L}^{-1}$ ,  $\text{KH}_2\text{PO}_4$  –  $0.1 \text{ g L}^{-1}$  Agar –  $20.0 \text{ g L}^{-1}$ , pH  $7.2 \pm 0.2$ . Colonies showing zone of hydrolysis were purified and grown on nutrient broth at  $37^\circ\text{C}$  for 48 h. After incubation the cell free broth was tested for fibrinolytic activity according to [Bajaj et al., \(2014\)](#). In brief,  $100 \mu\text{l}$  of supernatant was poured in well of fibrin agar plates composed of  $2.0 \text{ g L}^{-1}$  fibrinogen,  $0.2 \text{ g L}^{-1}$  thrombin (52 IU),  $\text{K}_2\text{HPO}_4$  –  $0.1 \text{ g L}^{-1}$ ,  $\text{KH}_2\text{PO}_4$  –  $0.1 \text{ g L}^{-1}$  and agar –  $20.0 \text{ g L}^{-1}$ , pH –  $7.2 \pm 0.2$ . Plates were then incubated at  $37^\circ\text{C}$  for 24 h. Isolates, which showed a clear zone around the colonies on fibrin agar medium, were isolated and used for the subsequent investigation.

To estimate the fibrinolytic activity,  $1.0 \text{ ml}$  fibrinogen solution ( $1.0 \text{ mg ml}^{-1}$ ) and  $0.1 \text{ ml}$  thrombin solution ( $1.0 \text{ mg ml}^{-1}$  corresponding to 52 units/ml) was added in centrifuge tubes and allowed to form clot. *In vitro* fibrin clot lysis assay was performed by dispensing  $100 \mu\text{l}$  cell free supernatant to centrifuge tube containing fibrin clot incubated at  $37^\circ\text{C}$  for 4 h. The fibrinolytic activity was calculated by percentage of clot dissolved. The quantitative estimation fibrinolytic activity was performed according to [PonnuSwamy et al. \(2014\)](#) with slight modification. In brief,

$100 \mu\text{l}$  cell free extract (as enzyme source) was added in glass tube containing clot and incubated at  $37^\circ\text{C}$  for 10 min in the water bath. The reaction was terminated by adding  $2 \text{ ml}$  of  $0.2 \text{ M}$  Trichloroacetic acid. The reaction mixture was transferred in centrifuge tube, after centrifugation the supernatant was analyzed for the fibrinolytic activity by recording the OD at  $275 \text{ nm}$  in a UV-Visible spectrophotometer. A fibrinolytic unit (FU) was defined as the amount of enzyme that gave an increase in absorbance at  $275 \text{ nm}$ , equivalent to  $1 \mu\text{g}$  of tyrosine per min at  $37^\circ\text{C}$ . The specific activity of the enzyme was calculated by estimating the total protein in the crude enzyme by Lowry's method.

### 2.3. Characterization of bacteria

Identification of biochemical and physiological properties of the selected microbe was done according to Bergey's manual of systematic bacteriology ([Brenner et al., 1984](#)) while molecular characterization was performed based on 16S rRNA gene sequencing. The bacterial genomic DNA was isolated by genomic DNA extraction kit (Sigma Aldrich). The 16S rRNA gene of the isolate was amplified by the use of forward primer 8F 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer 1492R 5' CCGTTACCTGTACGACTT 3'. The 16S rRNA gene sequence was determined by the Sanger's method (dideoxynucleotide chain termination method). The sequence was submitted to NCBI GeneBank under Accession number MK027134. Gene Sequence comparison with the databases was performed using BLAST through the National Center of Biotechnology Information server. The high scoring similar to 16S rRNA gene sequences were identified and aligned using CrustalW2 software. Phylogenetic tree was constructed using the neighbour-joining method in MEGA 7.2 software tool.

### 2.4. Effect of different physical and nutritional parameters on the production of fibrinolytic enzyme by one-factor-at-a-time approach

The effect of different fermentation related physiological parameters like incubation time (0, 12, 24, 36, 48 and 60 h), incubation temperature (24, 27, 30, 33, 36, 39 and  $42^\circ\text{C}$ ), initial pH of the production medium (5, 6, 7, 8, and 9), agitation (50, 100, 150, 200 and 250 rpm), concentrations of inoculum (1, 2, 3, 4 and 5%) and age of inoculums (12, 24 and 36 h) were investigated by one-factor-at-a-time (OFAT) approach. While different carbon sources (sucrose, dextrose, maltose, fructose, galactose, ribose, xylose, starch, dextran, cellulose) was analyzed by replacing dextrose, whereas various nitrogen sources (yeast extract, casein, tryptone, meat extract, soya meal, casitone, peptone,  $\text{NH}_4\text{Cl}$ ,  $\text{KNO}_3$ ,  $\text{NaNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$  was analyzed by replacing yeast extract in the medium (composed of dextrose –  $10 \text{ g L}^{-1}$ , yeast extract –  $10 \text{ g L}^{-1}$ ,  $\text{MgSO}_4$  –  $0.05 \text{ g L}^{-1}$ ,  $\text{KH}_2\text{PO}_4$  –  $0.05 \text{ g L}^{-1}$  and pH of 8.0) by one-factor-at-a-time approach. All the experiments were performed in triplicates and results were given as an average  $\pm$  standard deviation.

### 2.5. Selection of significant variables by Plackett-Burman design

Total seven variables dextrose, peptone,  $\text{K}_2\text{HPO}_4$ ,  $\text{MgSO}_4$ , inoculum level, age of inoculums and pH were selected for the experimentation. In PB experimentation, the best concentrations of these parameters obtained in OFAT were considered. The experimental design ([Table 1](#)) was generated using statistical designing tool Mintab14.2. The principal effects of each variable on fibrinolytic enzyme production were estimated as the difference between both averages of measurements made at the higher level and at the lower level. The significance level (P value) of each variable was determined using Student's *t*-test.

**Table 1**  
Plackett-Burman design for selection of enzyme production from *S. maltophilia* Gd2.

Variables Symbol Code Run order	Dextrose (% w/v)	Peptone (% w/v)	K <sub>2</sub> HPO <sub>4</sub> (% w/v)	MgSO <sub>4</sub> (% w/v)	Inoculum (% v/v)	Inoculum Age (hours)	pH	Enzyme activity (FU/ml)	
	X1	X2	X3	X4	X5	X6	X7	Observed value	Predicted value
1	2.2	1	0.08	0.02	1.5	12	8.5	1290	1324
2	2.2	3	0.02	0.08	1.5	12	6.2	1750	2096.56
3	0.6	3	0.08	0.02	4.5	12	6.5	1346	1410.67
4	2.2	1	0.08	0.08	1.5	24	6.5	870	606.72
5	2.2	3	0.02	0.08	4.5	12	8.5	3411	3114.78
6	2.2	3	0.08	0.02	4.5	24	6.5	1810	1815.67
7	0.6	3	0.08	0.08	1.5	24	8.5	2174	1743
8	0.6	1	0.08	0.08	4.5	12	8.5	1040	1219.94
9	0.6	1	0.02	0.08	4.5	24	6.5	811	797
10	2.2	1	0.02	0.02	4.5	24	8.5	1703	1919.94
11	0.6	3	0.02	0.02	1.5	24	8.5	2070	2038
12	0.6	1	0.02	0.02	1.5	12	6.5	702	496.72

### 3. Results and discussion

#### 3.1. Isolation of fibrinolytic enzyme producing bacteria and evaluation of *In-vitro* fibrin clot lysis

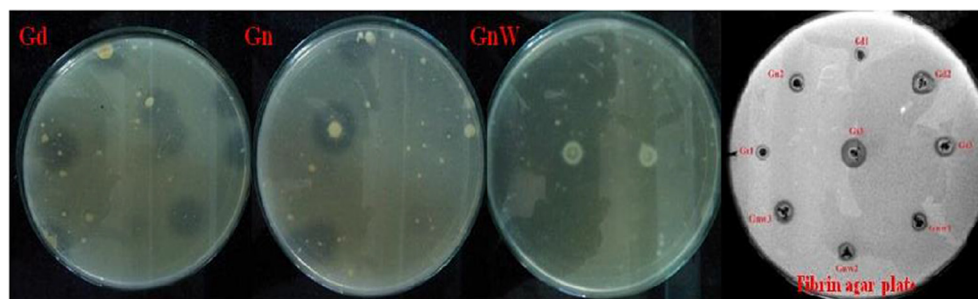
Selective isolation of fibrinolytic enzyme producing bacteria from the soil of Ganges (Gn) and Godavari (Gd) and water from Godavari (GnW) rivers yielded several protease producing microbial strains. Total 6 colonies with specific fibrinolytic activity were designated as Gd2, Gn1, Gn2, Gn3, GnW2 and GnW3 and selected for further investigation (Fig. 1). *In-vitro* fibrin clot lysis activity evaluation suggested that after 4 h a maximum of 74.88% and 64.92% clot lysis was observed in case of Gd2 and Gn3 isolates, respectively whereas less clot lysis activity 44, 43, 35 and 32% was observed in case of GnW3, Gn2, Gn1 and GnW2, respectively while Gd1, Gd3 and GnW1 does not show any lysis activity (Table 2 and Fig. 2). Since the clot was dissolved with cell free extract of isolates indicating the produced enzyme by these isolates belong to plasmin like protease family which directly act over fibrin. Critical evaluation of the experimental data from zone of clearance and clot dissolution further suggested that all proteases are not fibrin dissolvers hence, researchers working on fibrinolytic enzyme production should evaluate critically the fibrin lysis potential of proteases. A maximum of 856 FUml<sup>-1</sup> fibrinolytic activity (a specific activity of 36 Umg<sup>-1</sup> of protein) was observed for this Gd2 strain which is higher than many other reported fibrinolytic enzymes from *B. cereus* NS-2 (Bajaj et al., 2013), *B. subtilis* strain A1 (Jeong et al., 2004), *Bacillus* sp.KDO-13 (Lee et al., 2001) and *B. Subtilis* natto B-12 (Wang et al., 2009) which was ranging from 347 to 556 FUml<sup>-1</sup>. Since the strain Gd2 which was showing maximum activity hence further investigated.

#### 3.2. Characterization of bacterium

Morphological analysis of the selected bacterial strain Gd2 found to be Rod-shaped, Gram-negative, non spore-forming, motile and strictly aerobic. The bacterial colony appearance on nutrient agar plate was observed to be creamy coloured, circular, smooth and convex, margin was entire and texture was moist. Biochemical analysis indicated that this selected strain is catalase positive and utilize citrate as carbon source. The absence of acid and gas production upon its growth in presence of carbohydrates indicated that it does not ferment carbohydrates and produce acids indicating its inability to grow under anaerobic environment. Evaluation and comparative analysis of the data with Bergey's manual of systematic bacteriology, it was concluded that this isolated bacterium, Gd2 belongs to Xanthomonadaceae family and a member of *Stenotrophomonas* genus. Further identification of this isolate at species level was investigated based on molecular characterization 16SrRNA ribotyping by evaluating the genomic DNA of the isolate upon amplification and analyzing for molecular-based identification. A total of 562 base pair 16S rRNA gene was sequenced and submitted to NCBI GeneBank (Accession number MK027134). Blast analysis of the same denoted 98% similarity to *Stenotrophomonas maltophilia* family hence the isolate was designated *Stenotrophomonas maltophilia* Gd2. The phylogenetic tree which was constructed using MEGA 7 tool and by retrieving the sequences obtained in the BLAST search was reported in Fig. 3.

#### 3.3. Effect of different fermentation parameters on the production of fibrinolytic enzyme

The growth and any enzyme production is greatly influence by the various physiological parameter of the fermentation so it is



**Fig. 1.** Isolation of fibrinolytic enzyme producing bacteria from soil sample on casein and fibrin agar plate.

**Table 2**  
*In-vitro* fibrin clot lysis activity of selected isolates.

Sample	W1 (g)	W2 (g)	% clot dissolved $\pm$ SD
Gd1	1.12	1.12	0
Gd2	1.13	0.28	74.88 $\pm$ 1.57
Gd3	1.12	1.12	0
Gn1	1.11	0.72	35.14 $\pm$ 1.87
Gn2	1.13	0.64	43.48 $\pm$ 2.49
Gn3	1.13	0.40	64.92 $\pm$ 2.68
GnW1	1.12	1.12	0
GnW2	1.21	0.81	32.97 $\pm$ 1.59
GnW3	1.26	0.71	43.60 $\pm$ 2.48

\*W1: initial weight of clot, W2: weight of fibrin clot after incubation with enzyme.

necessary to evaluate those parameters. The effect of different fermentation related physiological parameters like incubation time, incubation temperature, initial pH of the production medium, agitation, level of inoculums and age of inoculums was investigated on fibrinolytic enzyme production by selected strain of *S. maltophilia* Gd2 by OFAT approach.

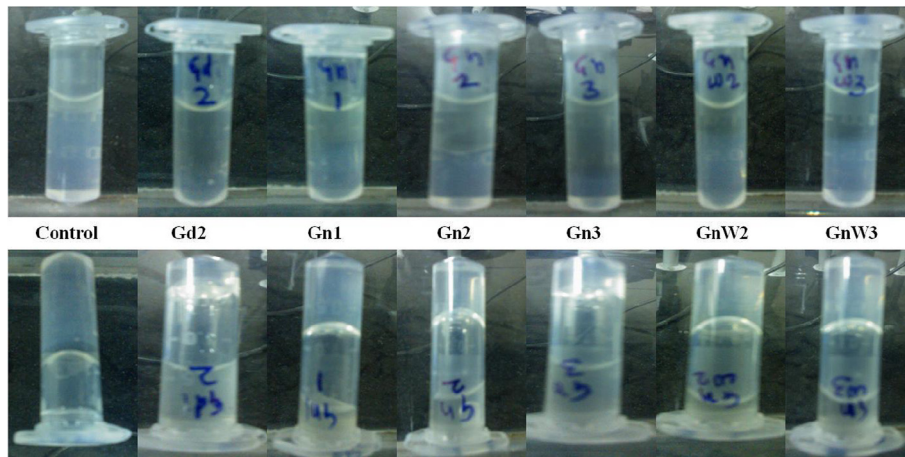
### 3.3.1. Effect of incubation time on fibrinolytic enzyme production

Effect of incubation time on fibrinolytic enzyme production from the selected strain *S. maltophilia* Gd2 was studied, the results suggested that the growth of the strain and enzyme production

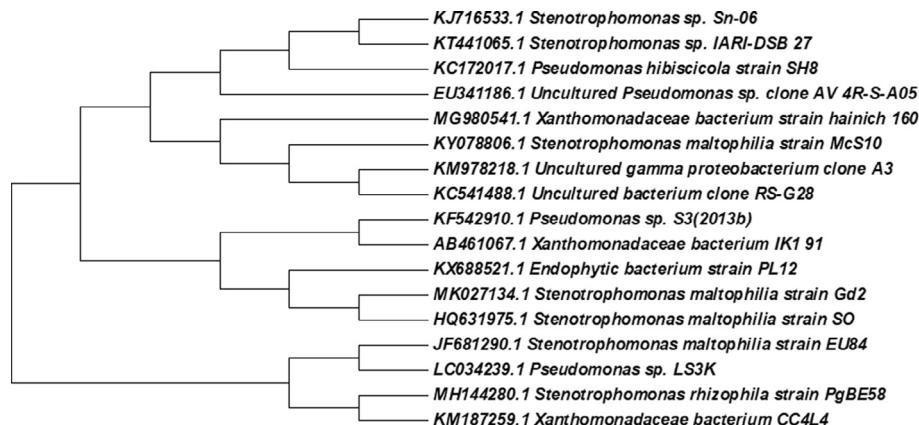
were increased with increase in incubation time indicating the fibrinolytic enzyme production in this strain is growth associated. A noticeable fibrinolytic enzyme production ( $234.1 \pm 12.5 \text{ FUml}^{-1}$ ) was observed at 12 h of incubation. The enzyme production reached to the maximum level ( $886.9 \pm 12.5 \text{ FUml}^{-1}$ ) at 36 h of incubation. The maximum rate of enzyme production was noticed in the log phase of growth (Fig. 4A). A constant enzyme production has also been observed during the stationary phase which further confirms that this enzyme production to this strain has nutritional importance for the growth of the strain.

### 3.3.2. Effect of initial pH

The nutrients transfer across the cell membrane as well as cellular metabolism is depends upon the pH of the surrounding which accountable for growth of the organisms as well as metabolite production. Hence, the effect of initial pH of the medium on extracellular production of fibrinolytic enzyme from *S. maltophilia* Gd2 at 37 °C on 150 rpm for 60 h was investigated at different pH environments (pH 5.0, 6.0, 7.0, 8.0 and 9.0) and analyzed for enzyme yields at 12 h interval. The data suggested that medium adjusted to pH 8.0 supported the growth of isolated *S. maltophilia* Gd2 with a maximum production enzyme ( $901 \pm 23.8 \text{ FUml}^{-1}$ ) (Fig. 4B) within 36 h of incubation which is almost three-fold that at acidic pH 6 ( $232.2 \pm 14.4 \text{ FUml}^{-1}$ ) and 6 times higher than that at pH5 ( $147.1 \pm 11.2 \text{ FUml}^{-1}$ ). The data also denoted that after 36 of



**Fig. 2.** *In-vitro* fibrin clot lysis activity of selected isolates. The liquid at the bottom of inverted tubes represents the clot dissolved while upper portion of same tube represents unresolved clot.



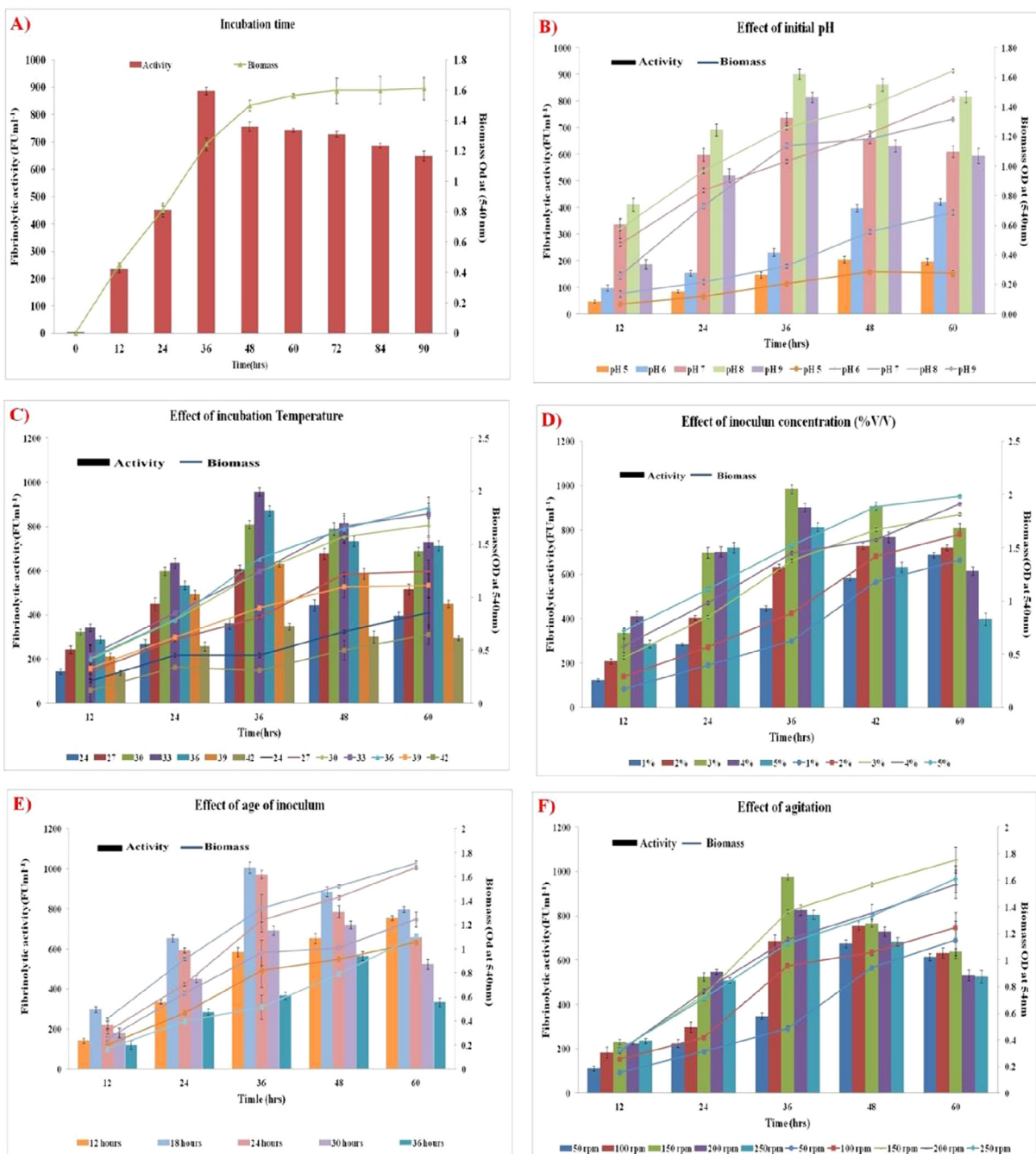
**Fig. 3.** Neighbor-joining Phylogenetic tree constructed according to Kimura two-parameter models is showing phylogenetic relationship of *Stenotrophomonas maltophilia* Gd2.

incubation the enzyme production has increased noticeably in alkaline pH conditions unlike acidic conditions. Similar trend of enzyme production at alkaline conditions was noticed (Mahajan et al., 2012; Agrebi et al., 2009; Bajaj et al., 2013).

### 3.3.3. Effect of incubation temperature

Incubation temperature plays pivotal role in the growth and metabolism of any microbe. However, the requirement of temperature varies with bacterium to bacterium and the rate of the metabolic reaction is largely influenced by temperature of fermentation. In fact, when the rate of metabolic reaction gets slow down at lower temperatures, in contrast incubation of the same microbial strain at higher temperature may responsible for the degradation

of the enzymes and increase in the fluidity of the cell membrane results in cell death. Considering the above the effect of temperature on *S. maltophilia* Gd2 growth and enzyme production was evaluated (Fig. 4C). The data revealed that *S. maltophilia* Gd2 produces biomass and fibrinolytic enzyme under all the experimental temperatures ranging from 27 to 42 °C suggesting this bacterium belongs to mesophiles class of the microorganism. Though enzyme production was observed in all the temperature environments, the optimum temperature was found to be 33 °C where the maximum enzyme yield was observed to be  $957 \pm 22.9$  FUml<sup>-1</sup>. Further rise in the temperature reduces the enzyme production, at 42 °C incubation temperature where the growth and enzyme production drastically reduced more than half ( $347.6 \pm 14.8$  FUml<sup>-1</sup>) which



**Fig. 4.** Effect of different Physical parameters on production of fibrinolytic enzyme from *S. maltophilia* Gd2. (A) Effect of incubation time (B) Effect of pH (C) Effect of incubation temperature (D) Effect of inoculum concentration (E) Effect of age of inoculums and (F) Effect of agitation.

suggested that 42 °C might be cardinal temperature for the *S. maltophilia* Gd2 above which growth of the organism was not observed. The maximum production of fibrinolytic enzyme by *Bacillus subtilis* I-2 (Bajaj et al., 2014) and Nattokinase by *Bacillus subtilis* (Fujita et al., 1993) was reported at 37 °C. However, thermophilic *Streptomyces* species reported to production fibrinolytic enzyme at 48 °C (Chitte and Dey, 2002).

3.3.4. Effect of inoculum concentrations

The inoculum concentration plays significant role in the production optimization as very less concentration of initial biomass may results in the long incubation time (long lag phase) whereas large inoculum level results in rapid increase in biomass ultimately results in nutrient stress affecting the product formation. Bacteria regulate their metabolism by gene regulation by the phenomenon called as quorum sensing. Quorum sensing is the type of cell signalling in which bacteria regulates their gene according the cell population in the surrounding (Eunhye et al., 2015). Therefore it is important to study the effects of various inoculum concentrations on the growth of microbial strain and associated product yield. The effect of inoculum concentration on the enzyme production by *S. maltophilia* Gd2 was investigated by inoculating 24 h grown culture to sterilized production medium (pH 8.0 ± 0.2) with different concentrations (1 to 5% v/v). The concentration of cells in the inoculum was adjusted such that it has optical density of 1.0 at

540 nm. After 60 h of incubation at 33 °C at 150 rpm, the samples were withdrawn and evaluated for the biomass and enzyme production. The results suggested that after 24 h of incubation there was increase in growth (OD at 540 nm 1.41 and 1.62) with respect to conditions where 4 and 5% inoculum was added as compare to that of 3% inoculums supplementation. Analysis of the enzyme production under these conditions indicated that the fibrinolytic activity (698.4 ± 24.25, 702.3 ± 22.09 and 721.1 ± 24.1 FUml<sup>-1</sup>) is almost same in the case of 3, 4 and 5% of inoculum concentrations, respectively after 24 h of incubation (Fig. 4D). In contrast to this after 36 h on incubation the less enzyme production and increased biomass was observed in 4 and 5% of inoculum as compare to that of 3% where a maximum production of 985.3 ± 20.3 FUml<sup>-1</sup> was found. With further increases in incubation time enzyme production slightly decreased in 3 to 5% while growth increase was observed. While there is constant increased in enzyme production as well as biomass was observed in case of 1 and 2% of inoculum. Addition of 2% inoculum concentration attained its maximum level (728.1 ± 14.1 FUml<sup>-1</sup>) of enzyme production after 48 h of incubation which is still less than that of 3% inoculums conditions where more than 150 FUml<sup>-1</sup> after 36 h of incubation. The results concluded that the 3% inoculum concentration was optimum for the maximum enzyme production of fibrinolytic enzyme from *S. maltophilia* Gd2. Any variation in either sides is reduces the enzyme yield.

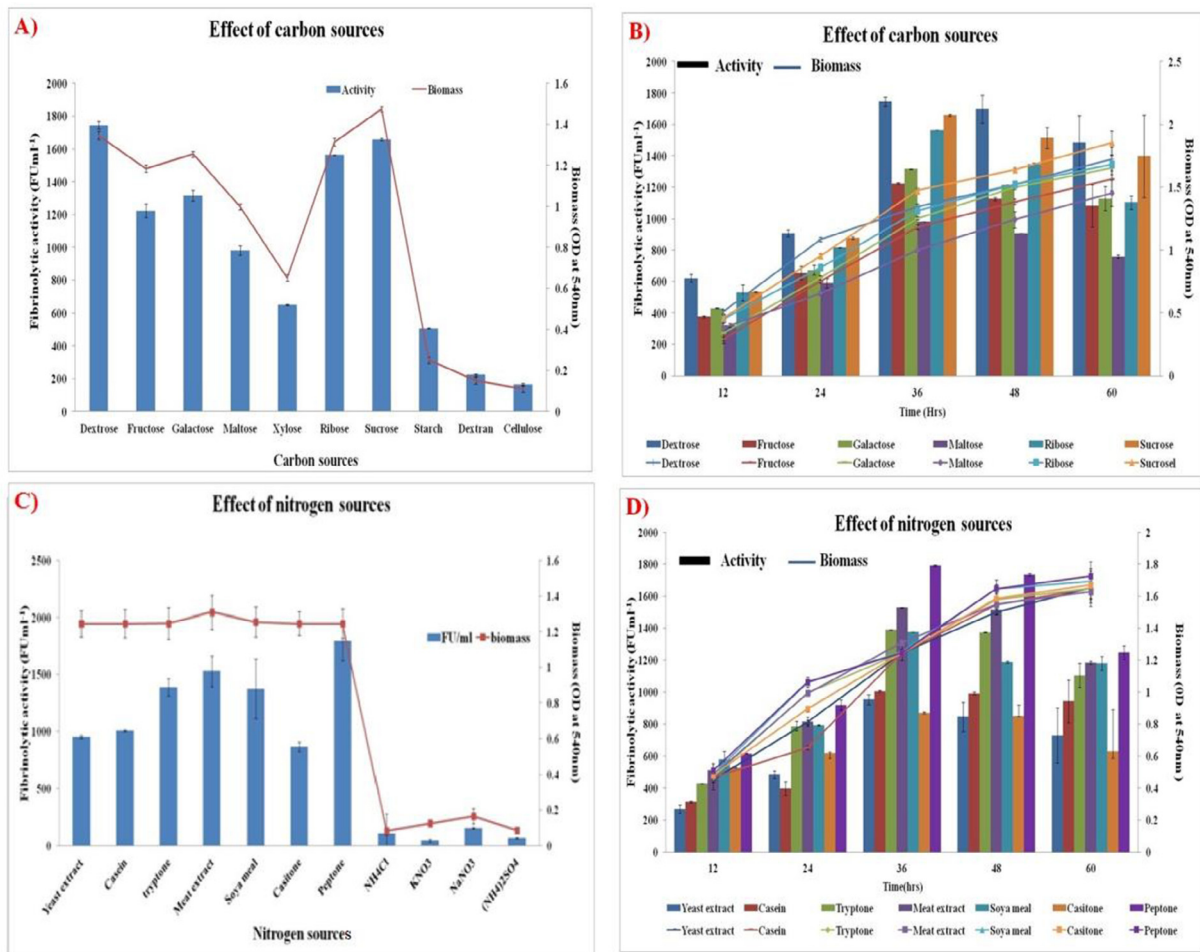


Fig. 5. Effect of different carbon and nitrogen sources on the fibrinolytic enzyme production from *S. maltophilia* Gd2. (A) Effect of different carbon sources after 36 h of incubation (B) Effect of carbon sources with respect to time (C) Effect of different nitrogen sources after 36 h of incubation and (D) Effect of nitrogen sources with respect to time.

### 3.3.5. Effect of age of inoculums and agitation

The other important physiological parameter in the fermentation optimization is age of inoculums as metabolic activity of the microbe differs with its growth phase. The inoculum should always be in log phase of growth where they bacteria divide rapidly which results in the higher yield with less incubation period, hence the effect of age of inoculums upon growth and enzyme production from *S. maltophilia* Gd2 were studied by inoculating seed culture of different age ranging from 12 h to 36 h. The fermentation experimentation was performed at 33 °C, 150 rpm for 60 h and the enzyme and biomass production was calculated at every 12 h of growth (Fig. 4E). The results suggested that maximum enzyme activity of  $1006 \pm 28.4$  FUml<sup>-1</sup> was observed after 36 h of incubation when the medium was inoculated with 18 h old inoculum which is almost similar with that of 24 h old inoculum  $972.4 \pm 19.5$  FUml<sup>-1</sup>. Further increase in age of inoculum imparts in reduced enzyme production. Whereas decreased age of inoculum required more incubation time. Fig. 4F revealed that the variation in the agitation speed (50 to 250 rpm) has played crucial role in all studied conditions. It was noticed that *S. maltophilia* Gd2 requires 150 rpm for maximum enzyme production ( $957.3 \pm 17.5$  FUml<sup>-1</sup>) and any variation resulted in negative regulation.

### 3.4. Effect of different carbon sources on the production of fibrinolytic enzyme

The carbon is basic element and essentially require for growth of the organism. In general, most of the microorganisms prefer the glucose as a carbon source hence primarily used as major carbon source for microbial growth and metabolites production. However, some bacteria will prefer another carbon source over the glucose. Considering the above, different carbon sources have been investigated to evaluate its impact on fibrinolytic enzyme production by *S. maltophilia* Gd2. Initially different carbon sources like sucrose, dextrose, maltose, fructose, galactose, ribose, xylose, starch, dextran and cellulose have been studied by replacing the dextrose in the fermentation medium with selected carbon source in the same concentration i.e. 1.0% (w/v) and by measuring the enzyme production after 36 h of incubation. The data represented in (Fig. 5A & B) revealed that the fibrinolytic enzyme production differed with type of carbon source indicating the metabolism of microbial strain differs with the carbon source and enzyme production however, dextrose as carbon source supported maximum enzyme production ( $1745.58 \pm 34.9.4$  FUml<sup>-1</sup>). Very least growth as well as enzyme production was observed with starch ( $508.6 \pm 16.4$  FUml<sup>-1</sup>), dextran ( $226.9 \pm 12.7$  FUml<sup>-1</sup>) and cellulose ( $165.3 \pm 28.4$  FUml<sup>-1</sup>), which indicated that the bacterium do not effectively utilize complex polysaccharides. The results also suggested that the *S. maltophilia* Gd2 can utilize the disaccharide (sucrose, galactose, and maltose) sole carbon sources, among these the sucrose shows higher production ( $1658.6 \pm 23.8$  FUml<sup>-1</sup>) as compare to galactose ( $1315.4 \pm 28.4$  FUml<sup>-1</sup>) followed by maltose ( $982.7 \pm 28.4$  FUml<sup>-1</sup>). It was also observed that fructose ( $1224.8 \pm 19.5$  FUml<sup>-1</sup>) and ribose ( $1562.7 \pm 21.4$  FUml<sup>-1</sup>) are also served as carbon source and supported enzyme production maximum at 36 h (Fig. 5B). This data is in accordance with reports of dextrose as a preferred carbon source (Fujita et al. 1993; Bhargavi et al., 2012). However, reports are also indicate increased fibrinolytic production with molasses (Bajaj et al., 2014), wheat bran (Agrebi et al., 2009) maltose (Bajaj et al., 2013) as a carbon source.

### 3.5. Effect of nitrogen sources

Nitrogen plays a major role in metabolism of the microorganisms which is required for the synthesis of biomolecules such as

amino acids, nucleic acids, proteins and enzymes. The microorganisms can utilize the nitrogen either in organic or inorganic in the form of salts. The fibrinolytic enzyme production largely depends on the availability of nitrogen sources in the medium, which has regulatory effects on enzyme synthesis. Although complex nitrogen sources were usually needed for proteases production, the requirement for a specific organic nitrogen supplement differs from organism to organism. High amount of protein containing organic nitrogen sources like yeast extract, casitone, meat extract, tryptone, casein, soya meal and peptone was used individually at 1% level in the media composed of dextrose –  $10 \text{ g L}^{-1}$ , nitrogen source –  $10 \text{ g L}^{-1}$ , MgSO<sub>4</sub> –  $0.05 \text{ g L}^{-1}$ , KH<sub>2</sub>PO<sub>4</sub> –  $0.05 \text{ g L}^{-1}$  and pH of  $8.0 \pm 0.2$ . The inorganic nitrogen sources such as KNO<sub>3</sub>, NaNO<sub>3</sub>, NH<sub>4</sub>Cl and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> showed little enzyme production by this strain (Fig. 5C).

Among the organic nitrogen sources, enzyme production ranged between 868 and 1795 Uml<sup>-1</sup> (Fig. 5D). Peptone supplementation showed a maximum production of  $1795.8 \pm 15.7$  FUml<sup>-1</sup> followed by tryptone  $1390 \pm 19.7$  FUml<sup>-1</sup>, soya meal  $1376.4 \pm 21.3$  FUml<sup>-1</sup>, casein  $1006.3 \pm 14.8$  FUml<sup>-1</sup> and casitone  $868.4 \pm 19.7$  FUml<sup>-1</sup> after 36 h of incubation. Yeast extract and soy/casein peptone reported to enhance the fibrinolytic protease production in *B. subtilis* ICTF-1 (Mahajan et al., 2012) and *B. amyloliquefaciens* An6 (Agrebi et al., 2010). Soybean powder and shrimp shell powder were reported as the best nitrogen sources for fibrinolytic protease production from *B. subtilis* LD8547 (Wang et al., 2008) indicating requirement for specific nitrogen source varies from organism to organism or even among the same species isolated from different sources.

### 3.6. Selection of significant variables by Plackett-Burman design

For the selection of the most significant variables for fibrinolytic enzyme production from *S. maltophilia* Gd2, a carbon, nitrogen sources, inorganic salts and cultivation parameters were tested and identified by the Plackett-Burman designed experiment. The design matrix selected for the screening of significant variables for protease production and the corresponding responses are shown in Table 1. After Plackett-Burman design of experiment, the enzyme production yield observed to be a maximum of  $3411 \text{ FU ml}^{-1}$  (Table 1 Run order 5) which is almost the double to that achieved through OFAT approach ( $1795 \text{ FU ml}^{-1}$ ). Same was confirmed by performing the validation experimentation. The competence of the model was calculated, and the variables evidencing statistically significant effects were screened via Student's *t*-test for ANOVA (Table 3). Factors demonstrating P-values of less than 0.05 were considered to have significant effects on the response, in other words lower probability values indicate the more significant factors on the production of fibrinolytic enzyme therefore can be selected for further optimization studies. Peptone and pH with a probability value of 0.006 and 0.019, were

**Table 3**  
Estimated effects and coefficients for enzyme activity (FUml<sup>-1</sup>).

Term	Effect	Coef	t	P
Constant		1582.9	16.57	0.000
Dextrose (X1)	445.5	222.7	2.33	0.080
Peptone (X2)	1021.2	510.6	5.35	0.006
K <sub>2</sub> HPO <sub>4</sub> (X3)	-322.5	-161.2	-1.69	0.167
MgSO <sub>4</sub> (X4)	186.2	93.1	0.97	0.385
Inoculum (X5)	207.8	103.9	1.09	0.338
Inoculum age (X6)	-19.8	-9.9	-0.10	0.922
pH (X7)	730.2	365.1	3.82	0.019

S = 330.870 R-Sq = 93.06% R-Sq (adj) = 80.91%.

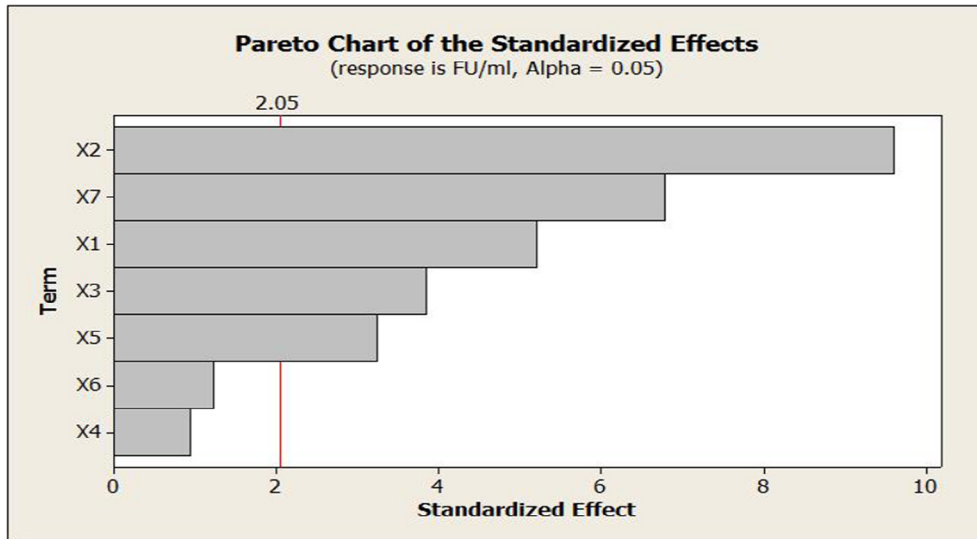


Fig. 6. Pareto chart for estimation of standardized effect of variables.

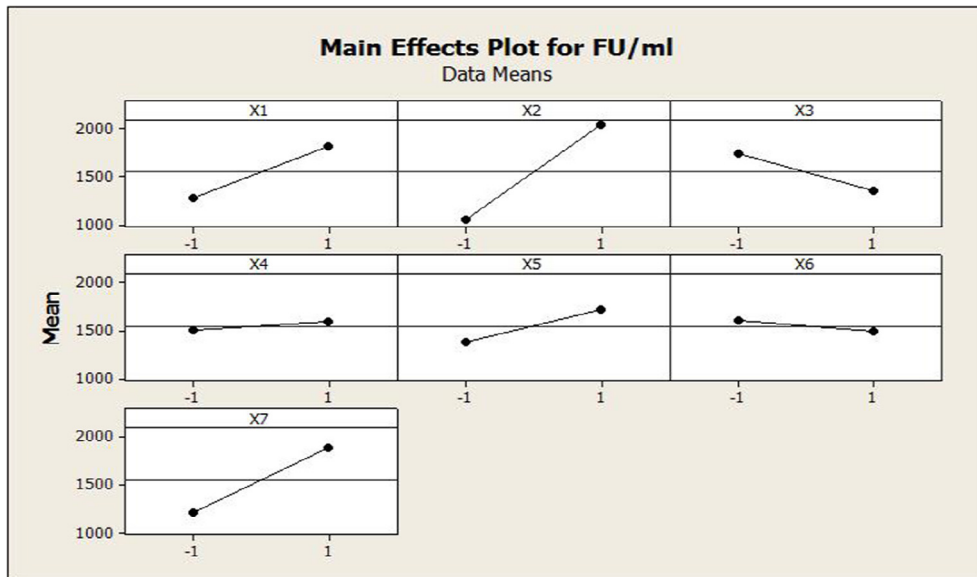


Fig. 7. Main effect plot for fibrinolytic enzyme production.

determined to be the most significant factors, followed by dextrose (0.080),  $\text{KH}_2\text{PO}_4$  (0.16), and inoculum concentration (0.338).

The main effect of each factor of experimentation can also be visualized by Pareto chart with alpha equals to 0.05 i.e. 95% of confidence level and 8 degree of freedom the t-value is equal to 2.02. The magnitude of each effect is represented by a column, and often, a line going across the columns indicates how large an effect has to be (i.e., how long a column must be) statistically significant to attend the 95% confidence. The results in the Pareto chart (Fig. 6) represent the standardized effect of the variables on the enzyme production. The standardized effects crossing the t-value 2.02 are significant. In the present study peptone, pH, dextrose,  $\text{K}_2\text{HPO}_4$  and inoculum concentration revealed significant effect on enzyme production while age of inoculum and  $\text{MgSO}_4$  did not show any significance indicating they can be neglected in the further optimization studies. Further the data represented in main effect plot (Fig. 7) indicated that dextrose, peptone, inoculum level and pH at higher level has significant effect on enzyme production in

contrast  $\text{KH}_2\text{PO}_4$  at lower level has significant effect, while  $\text{MgSO}_4$  and inoculum age does not show any significant effect on enzyme production.

#### 4. Conclusions

Overall, a new and novel fibrinolytic enzyme producing *S.maltophilia* strain designated as Gd2 was isolated from soil sample collected from Godavari river basin. This strain was characterized for its morphological, physiological and biochemical properties along with molecular level and identified as *Stenotrophomonas maltophilia*. This strain growth and fibrinolytic enzyme production pattern was studied by applying OFAT as well as PB model. The growth pattern and enzyme production regulated based on the nutritional and physiological environments. PB analysis depicted that dextrose as a carbon source and peptone as a nitrogen source and pH of the medium as well as inoculum level



have significant contribution on enzyme production. Considering the merit of this enzyme over other fibrinolytic enzymes, further studies will be focused on bulk production, purification, characterization at biochemical and kinetic levels followed by application potential to bring this to logical conclusion and possible utilization in health sector.

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