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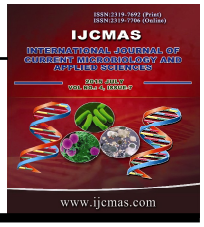

Original Research Article

Optimization of Medium Components for Enhanced Production of Extracellular Fibrinolytic Protease from *Citrobacter braakii*

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Keywords

Cardiovascular diseases (CVDs), fibrinolysis, fibrinolytic protease, C*itrobacter braakii*, optimization

A B S T R A C T

Cardiovascular complications due to thrombosis have become one of the major causes of mortality throughout the world. High cost and fatally undesired side effects associated with the available fibrinolytic agents to treat these diseases motivated the researchers to investigate potentially better agents for therapeutic applications. In the current investigation, production of an efficacious fibrinolytic protease from a bacterial isolate *Citrobacter braakii* was optimized by employing one factor at a time approach. Maximal yield of fibrinolytic protease was recorded with batch time 72 h, initial pH 7.0 and temperature 40°C. Sucrose, soya flour and di potassium hydrogen phosphate were found to be the best carbon, nitrogen, and mineral source, respectively for fibrinolytic protease production. Medium optimization resulted in 5.5 fold increased level of fibrinolytic production (198.6 $FUml^{-1}$) compared with that obtained with the initial medium (36.15 $FUmL^{-1}$). The present study advocates the use of *Citrobacter braakii* for large-scale production of fibrinolytic protease and application of produced enzyme as potential thrombolytic agent in pharmaceutical industries.

Introduction

Fibrin, which causes blood clotting, is activated, following fibrinogen degradation, by thrombin (EC 3.4.21.5), and can be lysed (fibrinolyzed) by plasmin (EC 3.4.21.7), a serine protease activated from plasminogen by tissue plasminogen activator (t-PA). Formation and fibrinolysis of fibrin maintained in balance by the homeostatic system (biological system); however, under an unbalanced situation due to some disorders, fibrin cannot be hydrolyzed. The accumulation of fibrin on the unbroken wall of blood vessels usually causes cardiovascular diseases (CVDs). Cardiovascular diseases, such as high blood pressure, acute myocardial infarction, ischemic heart diseases, valvular heart disease, peripheral vascular disease, arrhythmias, and stroke, are the primary causes of death worldwide (Mine *et al.*, 2005). In accordance with the data provided by World Health Organization (2012), by 2030 almost 25 million people will die from cardiovascular diseases (CVDs), mainly

from heart diseases and stroke. Drugs using fibrinolytic enzymes are the most effective methods in the treatment of CVDs. A variety of fibrinolytic enzymes such as tissue plasminogen activator (t-PA), urokinase (u-PA, EC 3.4.21.73), and bacterial plasminogen activator streptokinase (EC 3.4.99.22) have been the major players in the clinical setting all over the world for the treatment of these diseases (Moukhametova *et al.,* 2002). Although their efficacy remains undisputed, some limitations like excessive cost of clinical applications and the deleterious life-threatening side effects such as excessive bleeding and recurrence at the site of the residual thrombosis (Bode *et al.,* 1996), allergic reactions, difficulty in long-term use, low specificity to fibrin in the cases of urokinase and streptokinase, and short half-life of t-PA and urokinase have also been well documented. Because of the shortcomings of the available fibrinolytic drugs, attempts are underway to enhance the efficacy and specificity of fibrinolytic therapy and microbial fibrinolytic enzymes attracted much more medical interests during these decades. Various fibrinolytic enzymes were successively discovered from different microorganisms, including bacteria (Jeong *et al.*, 2015; Vijayaraghavan and Vincent, 2014; Majumdar *et al.*, 2014; Bhardwaj and Jayaraman, 2014; Bhargavi and Prakasham, 2013; Huang *et al.*, 2013), actinomyces (Ju *et al.*, 2012; Usegi *et al.*, 2011), fungi (Kumaran *et al.*, 2011; Rovati *et al.*, 2010), and algae (Choi *et al.*, 2013; Banerjee *et al.*, 2013).

Production of fibrinolytic enzyme by microorganisms differs qualitatively and quantitatively depending on the strains and species of microorganisms used as well as on their own nutritional and cultural conditions. The overall cost of enzyme production is one of the major challenges regarding the cost-effective industrial application of enzymes (Gupta *et al.*, 2002). In commercial practice, the optimization of the medium is done to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. So the optimization of medium components and cultural parameters is the primary task in a biological process.

In addition, no defined medium has been established for the optimum production of fibrinolytic protease from different microbial sources. Through traditional one-at-a-time optimization strategy, the individual effects of medium components can be seen on a graph without the need to revert to statistical analysis.

Considering the industrial values of fibrinolytic protease, in the present study, we tried to optimize the fermentation conditions for the production fibrinolytic enzyme from *Citrobacter braakii* (designated as *CbFP*) through one factor at a time approach.

Materials and Methods

Chemicals and reagents

Human fibrinogen and thrombin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Glucose, sucrose, xylose, lactose, melibiose, soluble starch, sodium citatre, peptone, sodium nitrate, casein, urea, soya flour, yeast extract, sodium chloride, dipotassium hydrogen ortho phosphate, magnesium sulphate, copper sulphate, ferric chloride, ammonium sulphate, calcium chloride, histidine, lysine, ornithine, arginine and trichloro acetic acid (TCA) was purchased from Himedia, Mumbai, India. Other chemicals were of analytical grade.

Microorganism and its maintenance

Citrobacter braakii (BGCC#2123) was procured from Bacterial Germplasm Collection Centre, 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. The production of *CbFP* was performed in basal medium containing (gL^{-1}) glucose, 10 g; yeast extract, 10.0 g and $K_2HPO4{\cdot}2H_2O$, 1.0 g with an initial pH of 7.0. At the end of fermentation period (24 h, 37°C), the culture medium was centrifuged at 10,000 rpm at 4°C for 15 min to obtain the crude extract, which served as enzyme source.

Fibrinolytic assay

Fibrinolytic activity was measured with a fibrin degradation assay developed by Japan Bio Science Laboratory and followed by Wang *et al.* (2009) 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.

Process optimization for maximum *CbFP* production

The various physicochemical parameters and media components required for maximum production of fibrinolytic protease by *Citrobacter braakii* were studied in 250 ml Erlenmeyer flasks at 150 rpm containing 50 ml of basal medium.

The media components and incubation parameters were optimized by using the 'one factor-at-a-time' approach wherein individual parameters were evaluated at a time keeping other parameters constant. The optimized parameter obtained (based on highest enzyme activity) was incorporated in the next experiment while optimizing the next parameters. The parameters included incubation period, pH, temperature, carbon, nitrogen, mineral ions and amino acids. All experiments were performed in parallel as triplicates.

Inoculum preparation

Inoculum was prepared in 250 ml Erlenmeyer flask containing 50 ml of aforementioned basal media of pH 7.0. The media was autoclaved at 121°C (15lbs) for 15 min and inoculated with a loopfull of 24 h old pure culture of *Citrobacter braakii*. The inoculated flask was incubated at 37°C in a shaking incubator (Remi C-24 BL) at 150 rpm for 12 h. From this culture, 1% inoculum was further transferred to 50 ml fresh sterile medium and further incubated at 37°C at 150 rpm for 12 h. This broth culture (A_{600} = 0.7) was served as seed inoculum for further experiments.

Biomass Yield: Bacterial biomass was determined by measuring the absorbance at 600 nm (Henroette *et al.*, 1993).

Effect of incubation period

The effect of incubation time on bacterial growth and *CbFP* production was determined by incubating the culture broth at different time interval (24-120 h) with an interval of 24 h and incubation period of 24 h was set as a control.

After incubation, the culture medium was centrifuged at 10,000 rpm for 15 min in cooling centrifuged at 4°C. Biomass and *CbFP* activity was determined.

Effect of pH

The effect of pH on bacterial growth and *CbFP* production was determined by adjusting the pH of the production medium in the range of 6-10 using 1N HCL and 1 N NaOH as appropriate. The medium with pH 7 was set as a control. Flasks were incubated at optimized incubation period at 37°C with shaking at 150 rpm and bacterial biomass and *CbFP* activity was determined.

Effect of temperature

The basal medium with optimum pH was inoculated with 1% seed inoculum and incubated at different temperature ranging from 25-45°C at 5°C interval for optimum period at 150 rpm. Incubation temperature of 37°C was set as a control and biomass and *CbFP* activity was determined.

Effect of Carbon sources

To determine the influence of different carbon sources on *CbFP* activity, various carbon sources (1.0%) (sucrose, xylose, melibiose, lactose, soluble starch and sodium citrate) were substituted in the medium in place of glucose. Biomass and *CbFP* activity was determined.

Effect of nitrogen sources

Various nitrogen sources like peptone, sodium nitrate, casein, urea and soya flour (1%) were examined for their effect on *CbFP* production by replacing yeast extract in the medium containing optimum carbon source. Biomass and *CbFP* activity was determined.

Effect of mineral salts

The effect of different metal ions (0.1%) viz. sodium chloride, magnesium sulphate, copper sulphate, ferric chloride, ammonium sulphate and calcium chloride was observed on growth and *CbFP* production by replacing dipotassium hydrogen ortho phosphate (control). Biomass and *CbFP* activity was determined.

Effect of Amino acids

To determine the effect of different amino acids (0.05 %) viz. histidine, lysine, ornithine, and arginine on growth and *CbFP* production, various amino acids were substituted individually and biomass and *CbFP* activity was determined.

Results and Discussion

Effect of incubation period

The culture broth was drawn on 24, 48, 72, 96 and 120 h of incubation to determine cell growth and CbFP activity (Fig. 1). The maximum *CbFP* production (50 $FUml^{-1}$) along with cell growth (A_{600} = 1.606) was obtained at 72 h of incubation.

The enzyme production and growth was gradually increases with increase in incubation period up to 72 h and then decreases with further incubation. Therefore,

incubation time selected for further experiments was 72 h.

Effect of pH

The classical approach for optimization of pH (6, 7, 8, 9 and 10) demonstrated maximum *CbFP* (66.3 $FUml^{-1}$) production from *Citrobacter braakii* at pH 7.0 whereas maximum growth of the bacteria was observed in medium of pH 8.0. Further increase in pH causes decrease in both the enzyme production and the growth with minimum at pH 10 (Fig. 2). Therefore, pH 7.0 was selected for further media optimization studies.

Effect of temperature

Citrobacter braakii was cultured in medium with optimum pH (pH 7.0) and incubated for optimum period (72 h) at different temperatures (25 ^{o}C, 30 ^{o}C, 37 ^{o}C, 40 ^{o}C and 50 ^{o}C). The results revealed that the maximum cell growth (A_{600} =1.936) was achieved at 37^{o}C while *CbFP* (67.5 $FUml^{-1}$) production was gradually increases upto 40^{o}C. At 45 ^{o}C the enzyme production was completely declined (Fig. 3). Therefore, temperature selected for further experiments was 40^{o}C.

Effect of carbon sources

Among the seven selected carbon sources (Glucose, sucrose, xylose, lactose, melibiose, soluble starch and sodium citatre), xylose was found to have maximum impact on growth (A_{600}= 3.427) of *Citrobacter braakii*, while maximum *CbFP* (79.3 $FUml^{-1}$) production was observed when sucrose was incorporated in the medium as sole carbon source, however incorporation of lactose resulted in decline of enzyme production (Fig. 4). Therefore, further studies were conducted using sucrose instead of glucose in the production medium.

Effect of nitrogen sources

In the present study, the organic and inorganic nitrogen sources were used to evaluate their ability for higher *CbFP* production. The results revealed that supplementation of soya flour in the basal medium as nitrogen source resulted in maximum *CbFP* (142.2 $FUml^{-1}$) production along with cell growth (A_{600} = 4.486).

Cell growth in presence of urea and sodium nitrate was very poor and no enzyme production was observed (Fig. 5). Therefore, further studies were conducted using soya flour instead of yeast extract in the medium.

Effect of mineral salts

Among seven tested mineral salts, maximum enzyme production (198.6 $FUml^{-1}$) along with cell growth (A_{600} = 1.930) was observed in presence of dipotassium hydrogen phosphate and significant *CbFP* production was also observed on incorporation of sodium chloride to soyaflour-based optimized medium (Fig. 6). Therefore, further optimization studies were conducted using dipotassium hydrogen phosphate in the medium.

Effect of amino acids

Five different amino acids (arginine, lysine, histidine, tyrosine and ornithine) were incorporated in the medium to observe their effect on growth and enzyme production. In presence of all the five amino acids *Citrobacter braakii* grew well but the production of enzyme was inhibited to varied levels, with maximum inhibition (18.22 fold) caused by histidine (Fig. 7).

In the present study, we report the optimization of various environmental and nutritional parameters for maximum production of fibrinolytic protease from *Citrobacter braakii.* The bacteria evidence its own idiosyncratic physicochemical and nutritional requirements for growth and enzyme secretion and there is no general defined medium for fibrinolytic enzyme production by different microorganisms. In view of the commercial utility of the enzyme, devising a cost-effective media formulation becomes a primary concern. In biotechnological enzyme production processes, even small improvements have been significant for commercial success (Reddy *et al.,* 2008).

Incubation period plays a substantial role in maximizing bacterial growth and fibrinolytic protease production. Optimal enzyme production was achieved at the stationary phase of the growth of *Citrobacter braakii* (BGCC#2123). An increase in 1.38 fold and 1.32 fold *CbFP* activity was observed at 72 hrs and 96 hrs respectively. After 120 hrs (i.e. 144 hrs data not shown), the enzyme production was completely inhibited. The probable reason for decrease in the *CbFP* production beyond 96 h may be due to rapid depletion of nutrients in the medium, accumulation of excess acid in the media as a result of sugar utilization and developed oxygen tension. Our results are comparable to Seo and Lee (2004), Wang *et al.* (2008) and Vijayaraghavan and Vincent (2014) who also reported maximum enzyme activity after 72 hrs from *Bacillus firmus* NA-1, *Bacillus subtilis* LD-8547 and *Paenibacillus* sp. IND8, respectively.

The microbial cells are significantly affected by the environmental pH because they in fact have no mechanism for adjusting their pH. It can affect growth of microorganisms either indirectly by affecting the availability of nutrients or directly by action on the cell surfaces. The metabolic activities of the microorganisms are sensitive to the pH changes and the pH of the culture media has marked effect on the type and amount of enzyme produced. Each enzyme has specific pH optima for its activity. Neutral media increased the protease production as compared to acidic or alkaline media (Rahman *et al.,* 2005). The maximum *CbFP* production (66.3 $FUml^{-1}$) was found at pH 7. This data was in accordance of Seo and Lee (2004) and Wang *et al.* (2009), who have also noted the highest yield of fibrinolytic protease at pH 7 with *Bacillus firmus* NA-1 and *Pseudomonas* sp. TKU015, respectively.

Temperature is one of the most critical parameter that has to be controlled in bioprocessing (Chi *et al.,* 2007). The temperature was found to influence bacterial extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane (Rahman *et al.,* 2005). An increase in 1.37 fold enzyme activity was observed at 40 ^{o}C and on exceeding the optimal temperature i.e. 45 ^{o}C, the enzyme activity was totally dropped most likely due to thermal denaturation of the enzyme. Our results are comparable to Kim *et al.* (1996) who also reported the maximum enzyme production at the same temperature while, the optimal temperature of *Streptomyces megasporus* SD5 for enzyme synthesis is 55 °C, because the strain was isolated from a hot spring (Chitte and Dey, 2002).

Maximum *CbFP* production (79.3 $FUml^{-1}$) with 1.09 fold increase was obtained when sucrose was used as sole carbon source. Enzyme activity was also found to be high on addition of sodium citrate (71.08 $FUml^{-1}$); as *Citrobacter braakii* is well known for utilization of sodium citrate as sole carbon source.

Fig.1 Effect of incubation period on growth and CbFP production

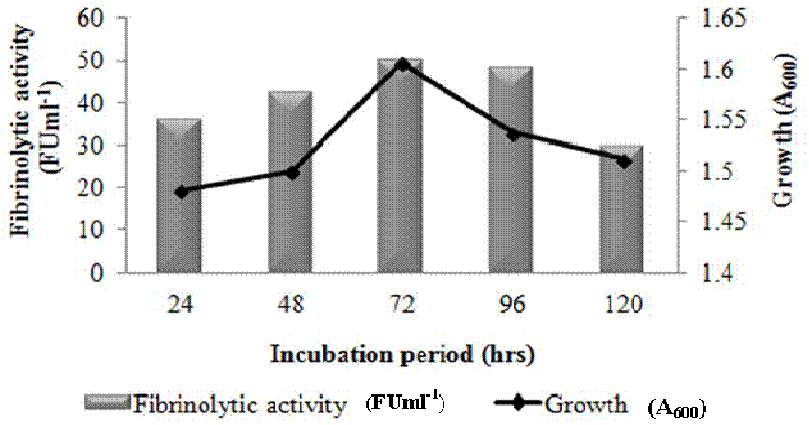


Fig.2 Effect of pH on growth and CbFP production

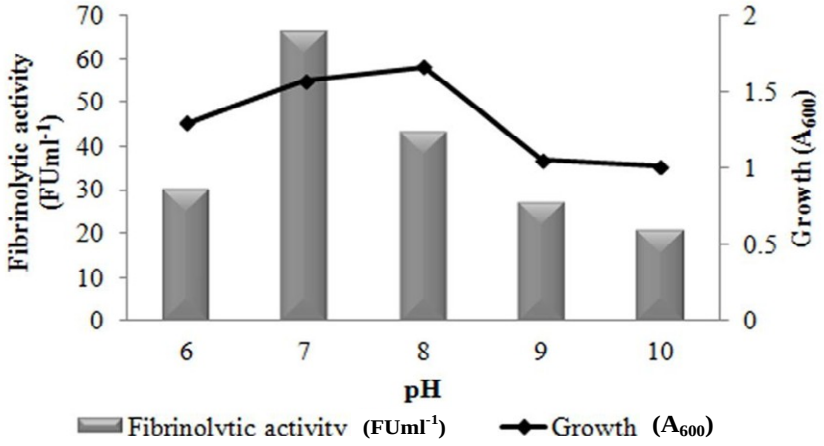


Fig.3 Effect of temperature on growth and CbFP production

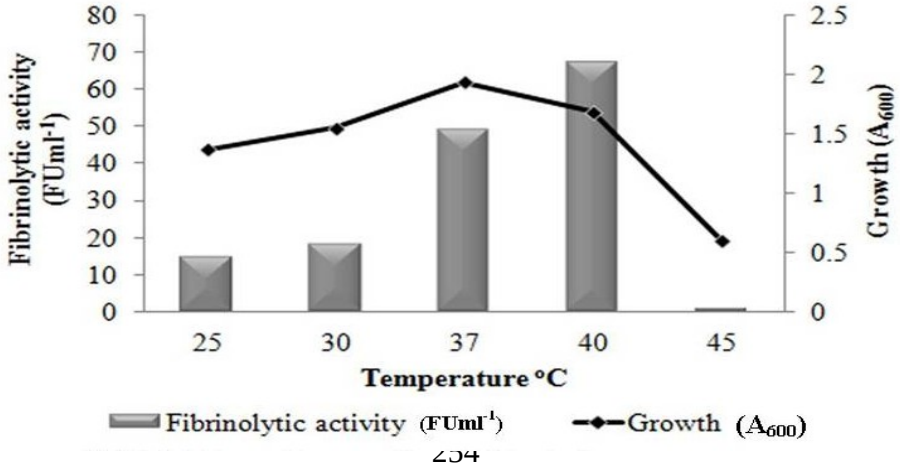


Fig.4 Effect of carbon source on growth and CbFP production

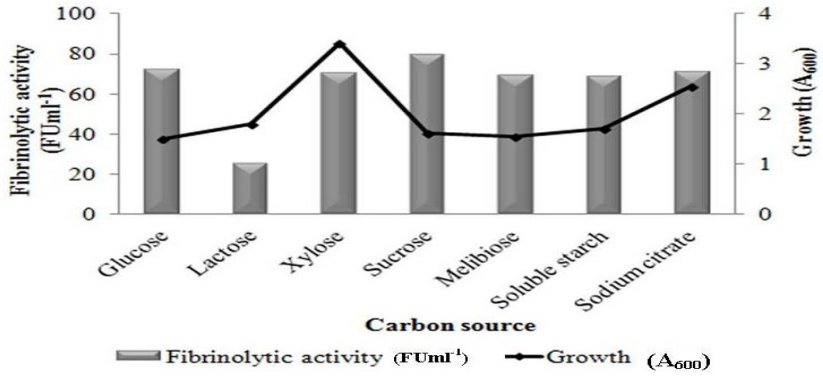


Fig.5 Effect of nitrogen source on growth and CbFP production

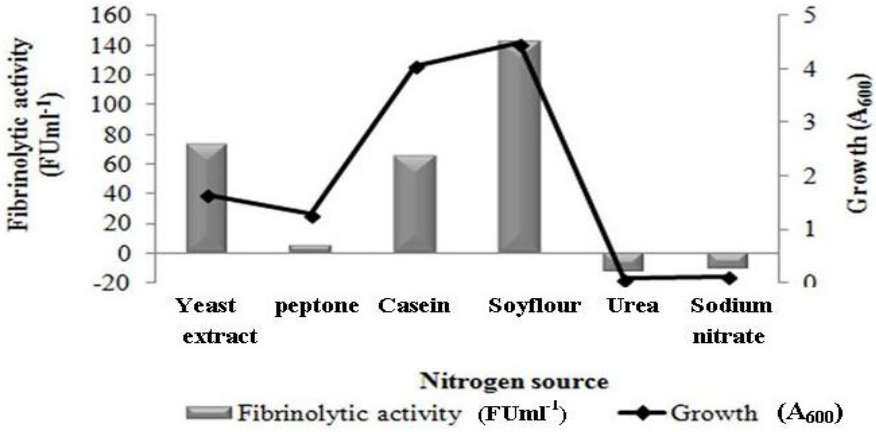


Fig.6 Effect of metal ions on growth and CbFP production

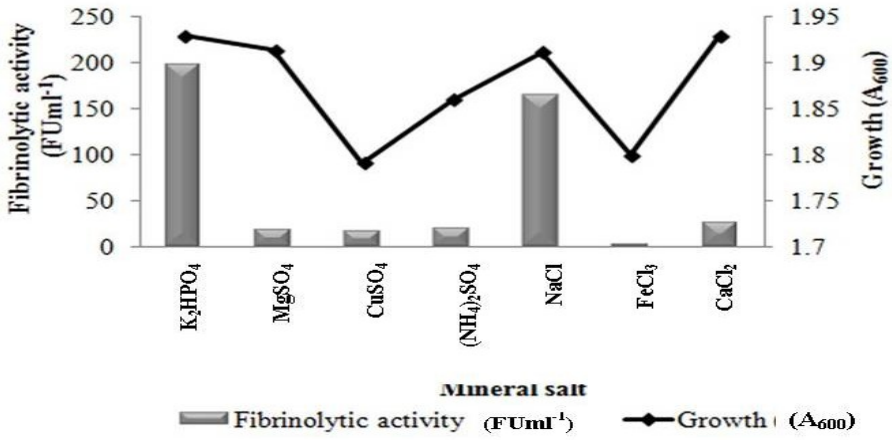
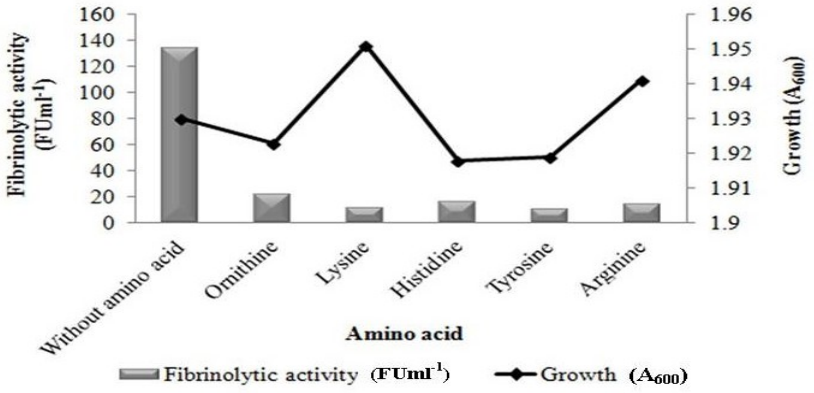


Fig.7 Effect of amino acids on growth and CbFP production



Liu *et al*. (2005) and Vijayaraghavan and Vincent (2014) also reported the positive effect of sucrose on fibrinolytic enzyme production from *Bacillus natto* NLSSE and *Paenibacillus* sp. IND8, respectively. Lactose played badly in this context. The repression effect of lactose was consistent with the findings of the study on alkaline protease production by *Bacillus* sp. (Chu, 2007).

Maximum *CbFP* production with 1.94 fold increase was obtained when soya flour was used as sole organic nitrogen source. This may be due to the enzyme catalysis reaction which could bring out glutamine and other amino acids from organic nitrogen sources. Glutamine is the precursor of nitrogen containing compounds and is one of the preferred nitrogen source involved in the metabolic pathway of *Citrobacter*. When inorganic nitrogen source (sodium nitrate) was incorporated to the medium no enzyme activity was found and growth of *Citrobacter braakii* was also very low. Fisher in 1991 reported that in biosynthesis, reduced form of nitrogen (as found in ammonium ions, amino groups or amide groups) is utilized and this may be the reason for the bad performance of sodium nitrate.

The trace elements and metal ions are one of the required cofactors for enzyme production. The present results confirmed that the dipotassium hydrogen phosphate (198.6 $FUml^{-1}$) and sodium chloride also induced the *CbFP* production. Inhibition of protease production by all the five amino acids tested revealed the possibility of catabolic repression of genes for protease production by rapidly metabolized amino acids in the medium. The catabolite repression by amino acids is a well known phenomenon in the yeast *Saccharomyces cerevisiae* (Jacob, 1999; Winderickx *et al.*, 2003). *Pseudomonas maltophilia* and *Bacilllus intermedius* also exhibited nitrogen catabolite repression, in which easily metabolized nitrogen sources repressed the expression of genes encoding transporters and proteases (Boethling, 1975).

From the present study it would be inferred that fibrinolytic protease production by *Citrobacter braakii* was enhanced upto 5.5 fold by optimization of environmental and nutritional parameters. The biosynthesis of *CbFP* from this strain was maximum when batch time 24 h, initial pH 7.0 and temperature 40°C was used. However, sucrose, soya flour and dipotassium hydrogen phosphate were best carbon,

nitrogen and mineral sources, respectively. The productivity can be further enhanced by using statistical method response surface methodology (RSM) using central composite design (CCD).Therefore, the fibrinolytic protease production by *Citrobacter braakii* emerges as a good alternative for further therapeutical application using biotechnology process.

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