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Utilization of sugarcane bagasse for enhancement production of fibrinolytic enzyme using statistical approach

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ABSTRACT

The purpose of this investigation was to produce fibrinolytic enzyme by utilizing sugarcane bagasse as low cost substrate through solid state fermentation in an effective way. The potential producer of microorganism, *Alcaligenes aquatilis PJS_1* was isolated and identified from slaughter house soil samples. In small scale level, higher production of microbial fibrinolytic enzyme was observed when the conditions provided like Glucose for carbon, Casein for nitrogen and $(NH_4)_2SO_4$ for mineral sources when maintained at 60 h with 100% moisture. The sugarcane bagasse medium with glucose and casein was found to have optimum production when incubated for 60 h at 35°C. The culture conditions were optimized to improve the production of fibrinolytic enzyme (3832 U ml^{-1}) from *Alcaligenes aquatilis PJS_1* through statistical approach. Blood clotting assay was performed to determine its anticoagulant property. The fibrinolytic property of the synthesised enzyme was confirmed when it dissolved blood clot sample effectively. Thus, the enzyme it could be used in medicine to produce as a thrombolytic agent.

1. Introduction

Sugarcane (*Saccharum officinarum*) is the widely grown agricultural crop in India. The sugarcane was crushed to get the cane juice and this generates nearly 270–280 kg of bagasse as agro waste per hectare (Hymavathi et al., 2010). In India, approximately 40 million metric tonnes of bagasse is generated every year and it was used as suitable raw material for production of value added products such as organic solvents, amino acids and compounds of pharmaceutical importance therapeutic enzymes because it is contain rich availability in hemicellulose (25%), lignin (25%) and cellulose (50%) approximately (Holmström et al., 1999). Likewise, Molasses and sugarcane bagasse can be employed as substrates for fibrinolytic enzyme production (Hmood and Aziz 2016).

Solid-state fermentation (SSF) and submerged fermentation processes were reported to produce many fibrinolytic agents which are currently available commercially to treat cardio vascular diseases (Basson, 2008). SSF was suggested to be the appropriate method to be used for the microbial fermentation, as it can take place in absence of free water, thus providing an environment similar to that of the natural habitat of the chosen microbes (Holmström et al., 1999). SSF process has many advantages such as convenient substrate usage, minimized pollution and high yield compared to submerged fermentation (Hmood and Aziz 2016). However, SSF has commercialized for numerous important pharmaceutical products production especially therapeutic enzymes. Some of the commonly used agents are prourokinase, urokinase, streptokinase, reteplase (r-PA), alteplase (t-PA) etc., (Brogden et al., 1973).

In General, the production of enzymes from microbial sources could be enhanced using optimum medium sources like carbon, nitrogen and mineral along with optimal environmental physical conditions such as period of incubation, temperature, pH and size of inoculum of the sample. The component that is responsible for the designing of production medium for bioproducts like enzymes cost is one of the curial factors in an industrial perspective, because the market price of the particular enzyme like bioproducts were increased based on their cost of production medium component. Hence, the utilization of locally available inexpensive substrates could bring down the production cost (Huang et al., 2012). Medium optimization is additionally a crucial think about enzyme bioprocess. Optimized media enhance the enzyme yield and reduce the assembly production cost. Using one-factor-at-a-time method in the optimization of parameters followed

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in the fermentation process was found to be considered; but still, it fails to give the information on the interaction between the factors or variables taken. The effective method is statistical optimization wherein the medium components having the foremost significant effect on bioprocess are often identified and optimized (Ju et al., 2012). Central composite rotational design (CCRD) and response surface methodology (RSM) elucidate the best-yielding concentration of every think about the fermentation process.

The number of variables that can be studied with lower number of experiments is more in RSM. This saves cost and time and makes RSM more effective than classical methods (Srinivas et al., 1994). RSM has been applied to various cases of enzyme production (Taneja et al., 2019).

The aim of this study is to produce the fibrinolytic enzyme by *Alcaligenes aquatilis PJS_1* using sugarcane bagasse as a solid substrate in a bench scale bioprocessing and to enhance fibrinolytic enzyme production by optimization of various culture conditions utilizing reaction surface methodology (RSM) with two-level full-factorial approach and fibrinolytic property of the enzyme was evaluated.

2. Materials and methods

2.1. Isolation, screening and identification of fibrinolytic enzyme producing bacteria

Soil samples were collected from different slaughter houses located in Karur district of Tamil Nadu, India. After serial dilution, the soil sample of 10^{-6} dilution was poured on nutrient agar plates by spread plate technique using L-rod. The isolated colonies were streaked on blood agar plates to check its fibrinolytic activity. All plates were incubated at 37°C for 24 h and a total of 10 positive isolates were obtained. The strains were further screened for casein hydrolysis (Gopinath and Lingappa 2016) and fibrin hydrolysis described by Astrup and Mullertz (1952). The strain which showed maximum zone in both casein and fibrin was selected for further studies as described by Kim et al. (2006). The colony was grown on nutrient agar plates repeatedly and stored at 4°C. Biochemical characterization and identification of the strain with BLAST using 16s rRNA sequence was performed as the similar work carried out by Mahajan et al. A sequence similarity search was performed using BLAST database in NCBI which showed 99 % similarity with *Alcaligenes aquatilis*. A phylogenetic tree was constructed using clustal W software to identify its closest relatives. The partial nucleotide sequence of the strain was deposited to GenBank database and the accession number MG952633 was allotted.

2.2. Parameter optimization studies

The production of fibrinolytic enzyme depends on several process parameters. These parameters vary for different microbial strains being employed for the production and hence parameters such as incubation time, pH, temperature, carbon, nitrogen and inorganic salts have been optimized to ensure maximum enzyme production. The production media was inoculated with 1 ml culture and incubated at different time intervals ranging from 12 h to 72 h. The effect of various carbon sources on the enzyme activity in the production medium was studied using Starch, Maltose, Sucrose, Glucose and Lactose at 1% concentration. Various nitrogen sources such as beef extract, yeast extract, peptone, casein, urea and gelatine at 0.5% were used. In addition, effect of various inorganic salts such as $CaCl_2$, $MgSO_4$, $FeSO_4$, Na_2HPO_4, NaH_2PO_4 and $(NH_4)_2SO_4$ at 0.1% were also assessed. The effect of temperature was studied at 15 °C, 25 °C, 35 °C, 45 °C, 55 °C and 65°C. Sugarcane bagasse substrate was collected from sugar industries at nearby Namakkal district of Tamilnadu, India. 5g of grounded dried sugarcane bagasse was taken. The initial moisture level was adjusted to 50%–100% by the addition of required amount of salt solution and 0.5% of casein and glucose was added. It was sterilized at 15 lb/in^2 for15 min.1 ml of *Alcaligenes aquatilis PJS_1* pure culture were inoculated into the medium. 50 ml of the culture medium in a 250 ml Erlenmeyer flask was incubated aerobically on a rotary shaker (120 rpm)at various conditions (i.e. temperature, moisture level and incubation period). At the end of the cultivation, the crude enzyme was obtained by centrifugation at 7000 rpm at 4°C for 10 min. Activities of the enzyme were determined by standard enzyme assay (Hariharan et al., 2014).

2.3. Statistical analyses

The variables that affecting the fibrinolytic enzyme production from novel microbe such as *Alcaligenes aquatilis PJS*_1 by using sugarcane baggase was determined by systemic design of different experiments using two level (2^5) full factorial design (Design Expert Software Version 12.0.0, Stat-Ease Inc., Minneapolis, MN, USA). Based on the preliminary studies minimum and maximum level of independent variables (Taneja et al., 2019) i.e., fructose, urea, ammonium sulphate were selected while all other variables were kept constant.

2.4. Fibrinolytic activity assay

Fibrinolytic enzyme was assayed by the hydrolysis of fibrin. The reaction mixture contained 2.5 mL of fibrin (1.2%, w/v), 2.5 mL of Tris-HCl (0.1 M) containing 0.01M $CaCl_2$ (pH 7.8), and a suitable amount of enzyme. The incubation was carried out for 30 min (37∘C), and the measurement time fell within the linear portion of the progress curve. The reaction was stopped by adding 5 mL trichloroacetic acid (TCA) containing 0.22M sodium acetate and 0.33M acetic acid. The absorbance of the supernatant was measured at 275 nm. One fibrinolytic enzyme unit was defined as the amount of sample that gave an increase in absorbency at 275 nm equivalent to 1 μg of tyrosine per min at 37°C (Vijayaraghavan *et al.* 2016).

2.5. In-vitro assay for blood clot lytic activity of fibrinolytic enzyme

About 0.5-ml aliquots of goat blood were collected from the slaughter shop at Namakkal District in Tamilnadu, India and allowed to form a clot. The other impurities that present on the blood clot was removed by washing thrice with phosphate-buffered saline and incubated with crude fibrinolytic enzyme. Positive and negative control of this experiment was prepared by using Streptokinase & phosphate-buffered saline. All tubes were incubated at room temperature for 6 h, and the results were observed for every 30 min (Vijayaraghavan et al., 2016).

3. Results and discussion

3.1. Isolation and identification for fibrinolytic enzyme production

In our study, a fibrinolytic enzyme producer bacterium *Alcaligenes aquatilis PJS_1* was chosen based on fibrin agar plate assay for production of anticoagulant agent (Collins and Lynee 1995). The *Alcaligenes aquatilis PJS_1* was identified based on biochemical tests and 16S rRNA gene sequencing and deposited in Gen Bank database under an accession number MG952633.

Likewise, microorganisms including *Bacillus subtilis* WR350 (Xiong et al., 2018), *Stenotrophomonas* sp. *KG-16-3* (Taneja et al., 2019), *Bacillus* sp. *IND7* (Vijayaraghavan et al., 2016), *Shewanella* sp. *IND20* (Vijayaraghavan et al., 2015), *Serratia* sp.*RSPB11* (Bhargavi and Prakasham 2013) from both land and marine environment have also been reported to produce various fibrinolytic enzymes. Production of fibrinolytic enzymes using Solid state fermentation from *Alcaligenesaquatilis PJS_1* was not reported so far and our results would be a promising source in future investigations.

3.2. Selection of fermentation condition by one variable-at-a-time (OVAT) approach

The recognised OVAT approach was used for medium formulation including carbon source, nitrogen source, mineral salts and environmental conditions such as incubation period, temperature and moisture content for enhancing the fibrinolytic enzymes production with sugarcane bagasse as potent substrate in solid state fermentation.

In this study, the production media was inoculated and incubated for time an interval ranging from 12 h to 72 h and the enzyme activity was noted every 12 h. From the results, it was observed that fibrinolytic enzyme production commenced and reached the maximal enzyme activity (1960 U ml^{-1}) in 60 h of incubation continued till 72 h and decreased thereafter (Fig. 1). For further experiment, the incubation period was maintained at 60 h. Our strain *Alcaligenes aquatilis PJS_1* has shorter period of incubation time for fibrinolytic enzyme production when compared to earlier reports. Vijayaraghavan et al. (2016) reported that the 72 h was optimized incubation time with *Bacillus* sp. IND7 for fibrinolytic enzyme production.

The effect of incubation temperature is a crucial factor to enhance the growth and metabolite synthesis of any nature microorganisms. Temperature requirements vary from bacterium to bacterium, however, and the rate of metabolic reaction is largely influenced by fermentation temperature. The study of the effect of temperature on fibrinolytic enzyme production was analysed (Fig. 2). The maximum enzyme activity was found at 35 °C (2010 U ml^{-1}). Further rise in the temperature reduces the enzyme production, at 65 °C incubation temperature where the growth and enzyme production drastically reduced more than half (122 Uml^{-1}). Similar fibrinolytic enzyme production was reported by Taneja et al., 2019.

The moisture content is the next most crucial factor that has to be optimized for enzyme production with crude substrate in the medium. In this investigation, the effect of moisture content in the solid substrate medium was assessed for improved production of fibrinolytic enzyme. The minimum fibrinolytic activity (733 Uml^{-1}) was observed with lower moisture content (50%), while the maximum (2156 Uml^{-1}) was observed at 100 % moisture content. Similar result was obtained in the investigation by Vijayaraghavan et al. (2016), where the maximum (100%) percentage of moisture content to achieve more fibrinolytic enzyme production (Fig. 3).

The medium components play a significant role in enhancing the enzyme production. Different carbon sources were employed to determine the production of extracellular fibrinolytic enzyme after 24 h incubation. The *Alcaligenes aquatilis* PJS_1 strain grew in all media however, the fibrinolytic activity was different in different media and glucose displayed the best carbon source. The maximal activity was detected with glucose (2382 U ml-1) (Fig. 4). An increased yield of fibrinolytic enzyme production from various carbon sources such as lactose (Saxena and Singh 2010), maltose, sucrose have been reported from other resources. It is reported that maltose when used as carbon source was found to maximize the enzyme production i.e., 201.2 U ml^{-1} followed by other commonly used carbon sources (Taneja et al., 2019).

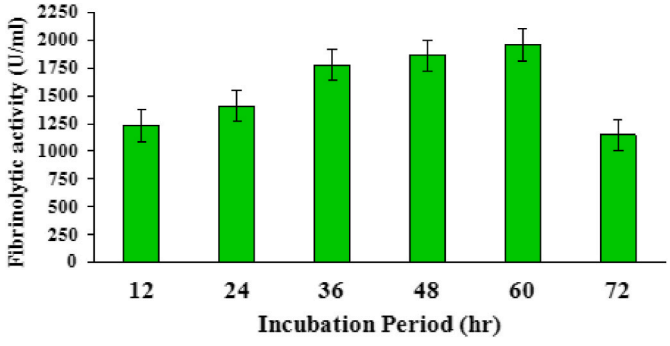


Fig. 1. Fibrinolytic enzyme activity for different incubation time (hr) from *Alcaligenes aquatilis PJS_1*.

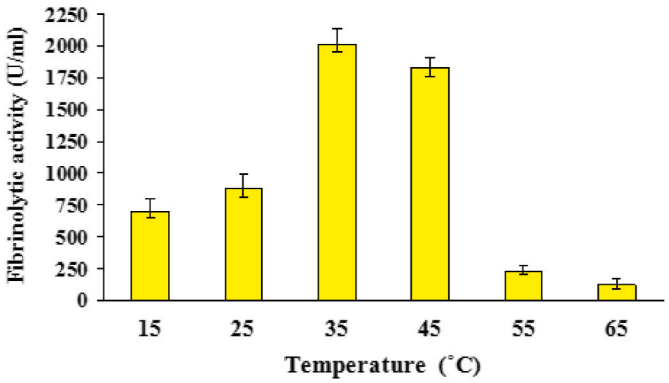


Fig. 2. Fibrinolytic enzyme activity for different temperature (°C) from *Alcaligenes aquatilis PJS_1*.

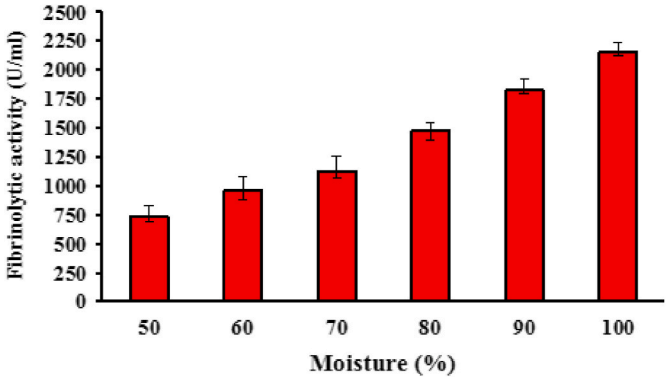


Fig. 3. Fibrinolytic enzyme activity for different Moisture (%) from *Alcaligenes aquatilis PJS_1*.

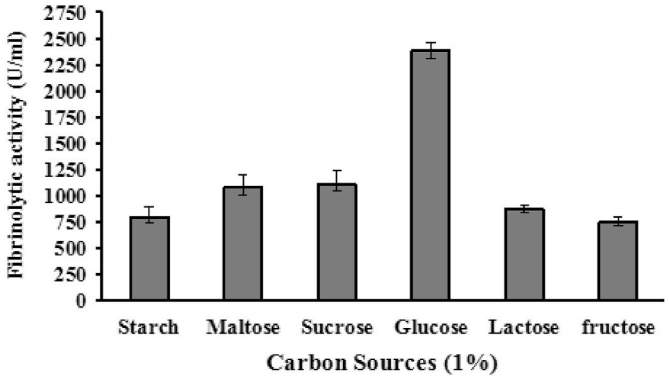


Fig. 4. Fibrinolytic enzyme activity for different Carbon sources, Nitrogen sources, and inorganic salts from *Alcaligenes aquatilis PJS_1*.

In general, nitrogen that are present in organic forms are metabolized to produce nucleic acids, amino acids, proteins and cell wall components (Wu et al., 2009).The effect of organic (beef extract, yeast extract, peptone, Casein, urea and Gelatin) nitrogen sources on fibrinolytic enzyme production by *Alcaligenes* sp. was investigated. It was noted that among different nitrogen sources, Casein (2178 U ml^{-1}) was found to be the best followed by Peptone (1310 U ml^{-1}) (Fig. 5). In earlier reports peptone (Venkataraman et al., 2010), casein, beef extract were found effective ingredients for enzyme production (Bajaj et al., 2014).

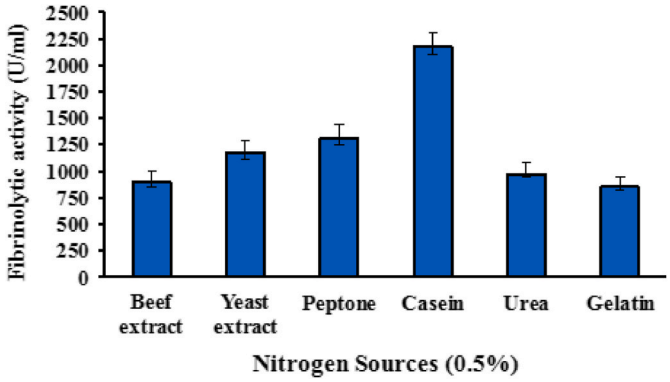


Fig. 5. Fibrinolytic enzyme activity for different Nitrogen sources *Alcaligenes aquatilis PJS_1*.

In the present study, the inorganic salts (NaH_2PO_4, $CaCl_2$, $MgSO_4$, Na_2HPO_4, $(NH_4)_2SO_4$ and $FeSO_4$) were evaluated for the production of fibrinolytic enzyme (Fig. 6). The medium was inoculated with 1 ml inoculum and incubated for 60 h interestingly, $(NH_4)_2$ SO_4significantly enhanced fibrinolytic enzyme production. In a previous report, NaH_2PO4, $MnCl_2$, $CaCl_2$ (Vijayaraghavan and Vincet 2014) was found to be the optimum inorganic salts for enzyme production. The presence of suitable concentration of metal ions in the production medium were plays a major role in serine protease production, because excess amount of their presence were inhibit the product during their translocation across the plasma membrane.

3.3. Screening of factors for the production of fibrinolytic enzyme by a two level full factorial design

Despite the conventional approach to medium optimization, i.e. in several cases, one-variable-at-a-time approach (OFAT) has been used successfully, it is repetitive, and often provide insufficient insight into process actions (Prafulla et al., 2012). To overcome the limitations of the conventional method, multivariate statistical technique is attracted by many researchers. Among the different optimization methods, the Response Surface Methodology (RSM) is the widely used method to estimate multiple variables.

Medium optimization by statistical analysis was used in the production of numerous

Compounds and in the fermentation process. In the present study, a two-level (2^5) full factorial design was employed to determine the optimum level of five process factors focusing for maximum fibrinolytic enzyme production. It gave more descriptive insight into the effect of each parameter on enzyme production along with their interactive

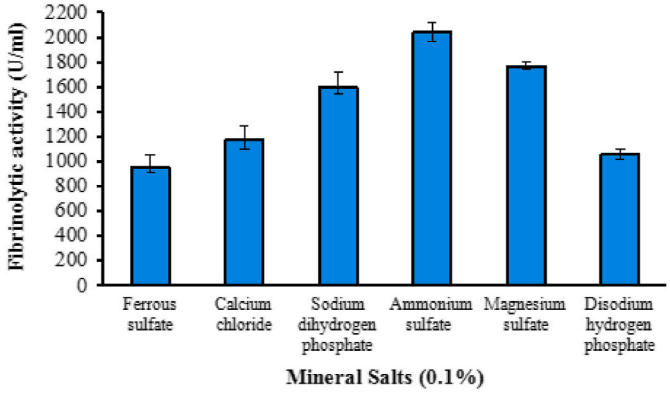


Fig. 6. Fibrinolytic enzyme activity for different inorganic salts from *Alcaligenes aquatilis PJS_1*.

effects with minimal experimental runs. Based on the OFAT studies, glucose as carbon source (%), Casein as nitrogen source (%), $(NH_4)_2$ SO_4 (%) as a mineral source, Moisture (%), temperature (°C) and incubation time (hr) were selected for this experiments. Thirty two experimental runs (Table 1) were carried out to study the effect of these five factors on fibrinolytic enzyme activity. The results were fitted to various polynomial models and subjected to ANOVA (Analysis of Variance) for assessment of their significance (Table 2). It was found that both the responses fitted well in the quadratic model without any transformation. The regression equation for polynomial models in terms of actual factors can be expressed by following Equation:

Fibrinolytic activity (U ml^{-1}) = + 1716.81 + 44.19 A - 24.06 B + 122.13C −177.62D + 130.38E + 1.69AB - 31.75 AC + 110.12AD + 401.50AE - 126.63BC - 133.13BD - 35.13BE −239.94 CD + 163.94 CE + 121.94 DE - 76.62 ABC - 273.50 ABD - 94.37ABE + 59.81 ACD + 342.81 ACE - 188.56 ADE - 41.81 BCD - 73.19 BCE - 74.69 BDE + 181.63 CDE - 89.19 ABCD - 143.69 ABCE - 140.56 ABDE - 119.88 ACDE + 20.87 BCDE

Where A-incubation Period, B-moisture, C-temperature, D-glucose and E-casein.

The production of fibrinolytic enzyme varied between 298 and 3832 U ml^{-1}. The traditional OFAT approach is simple, but it fails to explain the interaction between the variables. Hence, statistical methods of optimization are introduced to reinforce the assembly of fibrinolytic enzyme. Statistical approaches are wont to screen the variables and elucidation of optimum response of the variables (Box and Behnken 1960). Factorial experimental design is one such statistical procedure to review the foremost important component within the fermentation medium formula in enzyme bioprocess. The statistical methods such as Plackett– Burman (Prafulla et al., 2012), two-level full factorial design (Ahmad et al., 2013) is employed for the fibrinolytic enzyme production from *B. natto NLSSE*, *Bacillus* sp. *strain AS-S20-1*, and *B. cereus IND1*, respectively. Within the present study, using two-level full factorial design, a complete of 5variables were analysed in 32 experimental runs. Among the factors screened by statistical methods, time period, temperature and casein concentration of the medium significantly influenced the assembly of fibrinolytic enzyme. The three most vital factors that significantly influence the assembly of fibrinolytic enzyme were selected for optimization studies with RSM (Fig. 7).

The production media containing various concentrations of the components were gives the essential energy for the isolated microbes for huge level production of fibrinolytic enzyme. Through the optimization technique the cost of the fibrinolytic enzyme was decreased by optimizing the individual components that is required for their production from microbes. RSM may be a well-known statistical procedure that employs the cost-effective experimental design and has advantages like predicted response and evaluation. In an enzyme bioprocess, optimization for enhanced production is great challenge to develop feasible methods (Saravanakumar et al., 2010). Statistical optimization of fibrinolytic enzyme production was attempted by various researchers (Huang et al., 2013; Eldeen et al., 2015).However, no studies on optimizing variables in fibrinolytic enzyme production in SSF in RSM was carried out. To the best of our knowledge, this is the first study that deals the optimization of medium components for the production of fibrinolytic enzyme with sugarcane bagasse by *Alcaligenes aquatilis PJS_1* in SSF by statistical method. The R^2 of this model was 0.9967, which shows that this model was very effective in optimizing the selected individual factors and the present study showed good fit with this model design.

These studies imply that sugarcane bagasse contains significant amount of carbon and nitrogen sources for the production of fibrinolytic enzyme from *Alcaligenes aquatilis PJS_1*. The RSM-optimized culture medium enhanced fibrinolytic enzyme production than un-optimized medium. The optimization of fibrinolytic enzyme production by *Alcaligenes aquatilis PJS_1* of 3832 U Uml^{-1} achieved and demonstrating that sugarcane bagasse is an ideal culture medium for fibrinolytic enzyme

Table 1
Experimental design and results of 2^5 factorial design for fibrinolytic enzyme from *Alcaligenes aquatilis PJS_1*.

RUN	A -Incubation Period (hr)	B -Moisture (%)	C -Temperature (°C)	D -Glucose (%)	E -Casein (%)	Fibrinolytic activity (U ml^{-1})
1	1	−1	−1	1	1	1973
2	−1	1	1	1	1	1680
3	−1	−1	1	−1	−1	3832
4	−1	1	1	−1	−1	3120
5	−1	−1	1	−1	1	1523
6	1	1	−1	1	−1	1990
7	1	1	−1	1	1	1387
8	−1	1	−1	−1	−1	1645
9	1	1	1	−1	1	3253
10	1	1	1	−1	−1	1569
11	−1	−1	1	1	−1	1132
12	−1	1	1	1	−1	1022
13	1	−1	−1	1	−1	1783
14	1	−1	1	1	1	3892
15	−1	−1	−1	−1	1	1580
16	1	1	1	1	−1	298
17	1	−1	1	−1	1	3032
18	1	1	1	1	1	1383
19	−1	1	−1	1	−1	1648
20	−1	−1	−1	1	−1	1580
21	−1	1	−1	1	1	1648
22	1	−1	−1	−1	1	882
23	−1	1	−1	−1	1	1231
24	1	−1	−1	−1	−1	1321
25	1	−1	1	1	−1	842
26	1	−1	1	−1	−1	542
27	1	1	−1	−1	1	2541
28	1	1	−1	−1	−1	1488
29	−1	−1	−1	−1	−1	1571
30	−1	−1	−1	1	1	1247
31	−1	1	1	−1	1	1181
32	−1	−1	1	1	1	1122

Table 2
ANOVA results for the evaluation of significant factors by 2^5 factorial design.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.335E+07	30	7.784E+05	9.93	0.2469	**not significant**
A-Incubation Period	62481.13	1	62481.13	0.7969	0.5361	
B-Moisture	18528.13	1	18528.13	0.2363	0.7119	
C-Temperature	4.773E+05	1	4.773E+05	6.09	0.2452	
D-Glucose	1.010E+06	1	1.010E+06	12.88	0.1730	
E-Casein	5.439E+05	1	5.439E+05	6.94	0.2310	
AB	91.13	1	91.13	0.0012	0.9783	
AC	32258.00	1	32258.00	0.4114	0.6369	
AD	3.881E+05	1	3.881E+05	4.95	0.2689	
AE	5.158E+06	1	5.158E+06	65.79	0.0781	
BC	5.131E+05	1	5.131E+05	6.54	0.2372	
BD	5.671E+05	1	5.671E+05	7.23	0.2266	
BE	39480.50	1	39480.50	0.5035	0.6071	
CD	1.842E+06	1	1.842E+06	23.50	0.1295	
CE	8.600E+05	1	8.600E+05	10.97	0.1867	
DE	4.758E+05	1	4.758E+05	6.07	0.2455	
ABC	1.879E+05	1	1.879E+05	2.40	0.3651	
ABD	2.394E+06	1	2.394E+06	30.53	0.1140	
ABE	2.850E+05	1	2.850E+05	3.63	0.3075	
ACD	1.145E+05	1	1.145E+05	1.46	0.4401	
ACE	3.761E+06	1	3.761E+06	47.96	0.0913	
ADE	1.138E+06	1	1.138E+06	14.51	0.1634	
BCD	55945.13	1	55945.13	0.7135	0.5535	
BCE	1.714E+05	1	1.714E+05	2.19	0.3786	
BDE	1.785E+05	1	1.785E+05	2.28	0.3726	
CDE	1.056E+06	1	1.056E+06	13.46	0.1694	
ABCD	2.545E+05	1	2.545E+05	3.25	0.3226	
ABCE	6.607E+05	1	6.607E+05	8.43	0.2112	
ABDE	6.323E+05	1	6.323E+05	8.06	0.2156	
ACDE	4.598E+05	1	4.598E+05	5.86	0.2493	
BCDE	13944.50	1	13944.50	0.1778	0.7459	
Residual	78408.00	1	78408.00			
Cor Total	2.343E+07	31				

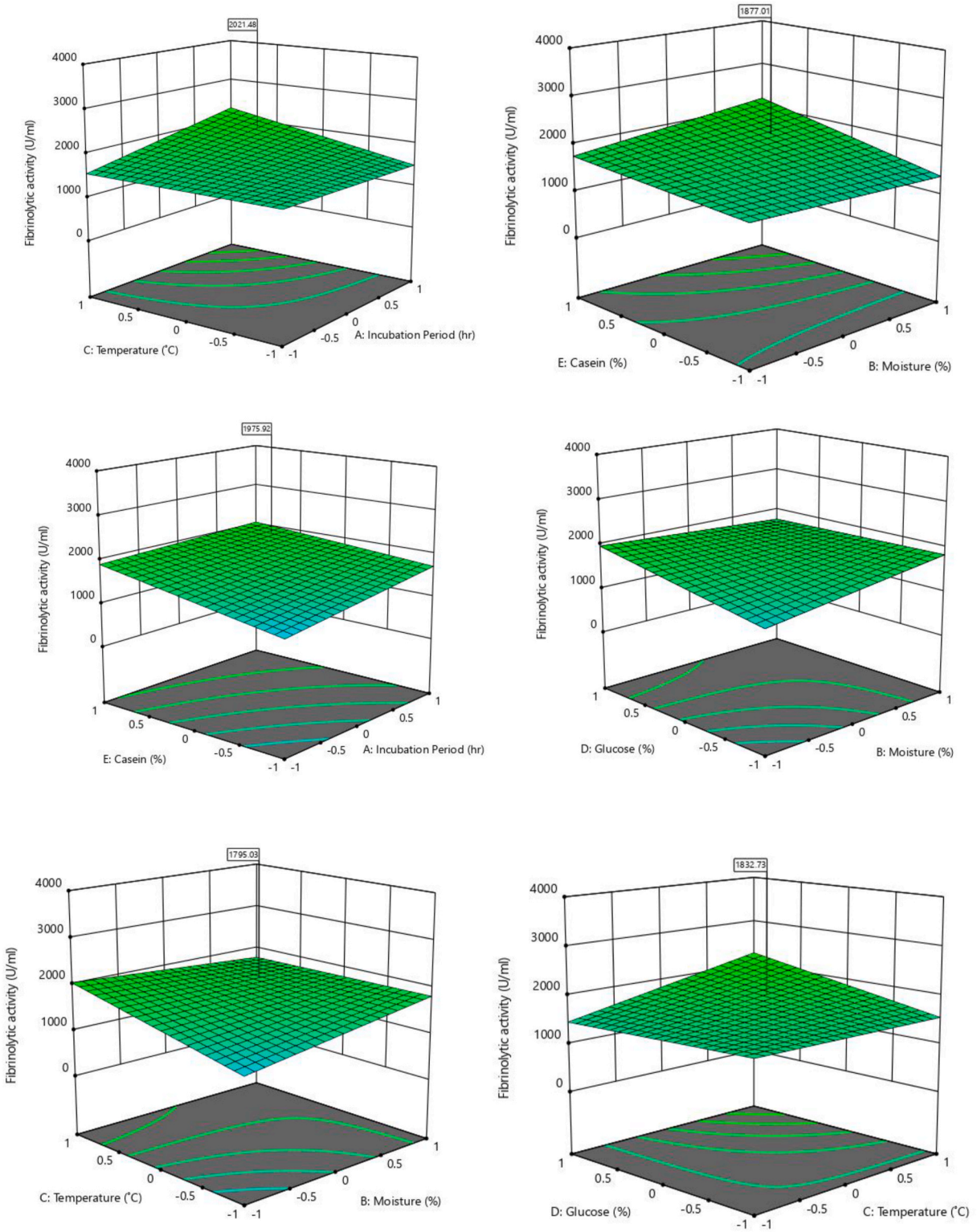


Fig. 7. Response surface plot for fibrinolytic enzyme production from *Alcaligenes aquatilis* PJS_1 in solid state fermentation. (a) The interactive effects of temperature and Incubation period; (b)interactive effects of casine and moisture; (c) interactive effects of casine and Incubation period; (d) interactive effects of Glucose and moisture; (e) interactive effects of temperature and moisture; (f) interactive effects of Glucose and temperature.

production from *Alcaligenes aquatilis PJS_1*.

Optimization of fibrinolytic enzyme production for solid state fermentation employs *B. subtilis HA*, *B. subtilis natto* (Pan et al., 2019), *B. subtilis* (104.9 U/g) (Huang et al., 2013) and *B. amyloliquefaciens* (Lee et al., 2010) demonstrating that sugarcane bagasse is an ideal culture medium for fibrinolytic enzyme production from *Alcaligenes aquatilis PJS_1*.This study demonstrated the application of a multifactorial statistical approach for determining a suitable fermentation conditions for the maximum yield of fibrinolytic enzyme production from a newly isolated strain such as *Alcaligenes aquatilis PJS_1*.

Anti-coagulant property can be determined when the enzyme is allowed to dissolve the coagulated blood in suitable incubation time

with specific pH and temperature. It is the easiest and preliminary step to check anticoagulant nature of any fibrinolytic enzyme extracted from microbes through this method. 200 μl of the purified enzyme obtained after chromatography was taken for anticoagulant test. The sample was added to a small amount of blood clot in a test tube. Then it was incubated and maintained for 2 h at 37°C. After the incubation time it was observed that, the blood clot was dissolved completely by the enzyme. It shows, the anti-coagulant property of enzyme obtained from *Alcaligenes aquatilis PJS_1*.

4. Conclusion

Based on the outcomes obtained in this investigation we were able to find new microbial strains for production of fibrinolytic enzyme with high ability an anticoagulant agent. This is the first report for fibrinolytic enzyme using sugarcane bagasse under solid state fermentation using *A. aquatilis PJS_1*. In the present study, we have isolated *A. aquatilis PJS_1* from slaughter house soil and investigated the optimal media components for the maximum fibrinolytic enzyme production. The optimum incubation time, temperature, inorganic salts, carbon and nitrogen sources for fibrinolytic enzyme production was found to be 60 h, 35°C, ammonium sulphate, Glucose and casein, respectively. The culture conditions were optimized to improve the production of fibrinolytic enzyme from *Alcaligenes aquaqtilis PJS*-1 by statistical approach. Further research includes the clinical trials of the enzyme as a potential fibrinolytic agent with the model organism proceeded by study of the pharmacological properties such as pharmacokinetics and pharmacodynamics. This can lead to production enzyme which has efficient clinical property in a large commercial scale for public health. Hence it has wide industrial potential besides its pharmaceutical importance and also *Alcaligenes aquatilis PJS_1*would be a promising resource in medical biotechnology due to its fibrinolytic action and further investigations on purification is needed. In conclusion, sugarcane bagasse is a promising agro-waste act as resource for the production of fibrinolytic enzyme which shows medicinal relevance.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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