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ORIGINAL RESEARCH ARTICLE

Isolation and Optimization of Streptokinase Production by Batch Fermentation

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ABSTRACT

Streptokinase was isolated from β haemolytic streptococci, *Streptococcus pyogenes* by batch fermentation. Strain was treated with U.V radiation. Enzyme concentration was measured by Lowry's protein estimation method. Regression analysis was performed to study the influence of different parameter like ageing period of strain, inoculum volume, corn steep liquor on the yield of enzyme. Biological activity like Fibrinolytic activity, Radial Caseinolytic activity was performed to study enzyme activity. M1 (45.04 mg/ml) batch was considered as a prominent batch as it produce almost five times higher yield than normal strain (9.68 mg/ml). Batch F6 (296.67 mg/ml) of factorial experiment was found to be most prominent for production of streptokinase enzyme as it shows almost six fold increments in production yield. Regression analysis stated that Corn steep liquor and ageing period of strain are the parameters which have positive significant effect on enzyme yield while inoculum volume has negative significant effect on yield.

Key words: Corn steep liquor, Streptokinase, U.V.Treatment, Factorial design, Yield

INTRODUCTION

A blood clot (thrombus) developed in the circulatory system can cause vascular blockage leading to serious consequences including death. A healthy haemostatic system suppresses the development of blood clots in normal circulation, but reacts extensively in the event of vascular injury to prevent blood loss. Outcomes of a failed haemostasis include stroke, pulmonary embolism, deep vein thrombosis and acute myocardial infraction. Pathologies involving a failure of haemostasis and the development of clot require clinical intervention consisting of intravenous administration of agent [1]. Streptokinase is one such agent. Other thrombolytic or Fibrinolytic agents include urokinase and the tissue type plasminogen activator (tPA) [2].

Microorganisms are capable of growing on a wide range of substrates and can produce a remarkable spectrum of products. The relatively recent advent of *in vitro* genetic manipulation has extended the range of products that may be produced by microorganisms and has provided new methods for increasing the yields of existing ones. The commercial exploitation of the biochemical diversity of microorganisms has resulted in the development of the fermentation industry and the techniques of genetic manipulation have given this well-established industry the opportunity to develop new processes and to improve existing ones [3].

A full factorial design may also be called a fully crossed design. Such an experiment allows studying the effect of each factor on the response variable, as well as the effects of interactions between factors on the response variable. For the vast majority of factorial experiments, each factor has only two levels. For example, with two factors each taking two levels, a factorial experiment would have four treatment combinations in total, and is usually called a *2×2 factorial design*. If the number of combinations in a full factorial design is too high to be logistically feasible, a fractional factorial design may be done, in which some of the possible combinations (usually at least half) are omitted [4].

The main objectives of present study was production of streptokinase from mutant species with used of corn steep liquor as nitrogen source and other essential elements to carry out fermentation process and study the influence of medium composition, inoculum volume and internal processing parameter like incubation time

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on the yield of enzyme by factorial design of experiment.

MATERIALS AND METHODS

Materials

Streptococcus pyogenes-MTCC 1923 was obtained from Microbial Type Culture Collection Chandigarh, maintained on nutrient broth medium and preserved at 4°C. Corn steep liquor was purchased from sigma chemicals, Bombay. All other reagents and biochemicals used in study are of high purity standard.

Fermentation process

The bacteria were cultured in nutrient broth medium at 37°*C*. By increasing the turbidity to the level of OD=0.6 at 600 *nm*, it was sub-cultured in 250 ml of broth; It was observed that the optimum pH for cell growth and streptokinase activity was at the neutral condition (pH=7).The culture was centrifuged for 25 minutes at 10,000 *g*. Prior to addition of solid ammonium sulphate to a final concentration of 65% (w/v), the supernatant was filtered through a 0.45 µm cellulose acetate filter. After standing at 4°*C* overnight, the precipitate was harvested by centrifugation at 4°*C* for 20 minutes at 12,000 *g* and dissolved in 1 *ml* of 10 mM Tris buffer, pH=8.0. As per above procedure streptokinase was isolated from six different batches. From that, normal strain was used in three batches and for the rest, U.V treated strain was used [5]. Preliminary batch was performed by designing six batches.

From six primary batches streptokinase enzyme was isolated and its concentration was measured by Lowry method. Optimized batch was further proceeding by using factorial design.

Factorial experiments:

2^3 Factorial designs were prepared. Three variables and two levels were used to design the experiment. The amounts of ingredients used in factorial batches were described in (**Table 1**).

Streptokinase assay

Streptokinase assays rely on its ability to activate plasminogen to plasmin. Plasmin then hydrolyses an indicator substrate and the extent of hydrolysis over a given period is related back to the concentration of streptokinase. The indicator substrates for plasmin include the fibrin clot, casein, other proteins and various synthetic esters (e.g., lysine methyl ester, lysine ethyl ester, ptoluenesulfonyl- L-arginine methyl ester).

Radial Caseinolytic activity

The skim milk agar medium was prepared and 100 µl of human plasma was placed on medium. Then wells were punctured in agar plate. 100 µl of streptokinase was loaded into the wells and kept for 12 hrs incubation at 37^0 C. After incubation zone of inhibition was measured and compare with standard streptokinase [6].

Fibrinolytic activity

Fibrinolytic activity was determined by the fibrin plate method. The fibrin plate was made up of 9 ml firinogen solution (1.5% bovine fibrinogen in 20 mM Tris–HCl buffer, pH 7.4), 2 ml of thrombin solution (1 BP U/ml in the same Tris–HCl buffer) and 9 ml of 1% Agarose in Petri dishes (5.5 cm in diameter) and 100 µl human plasma. The clot was allowed to stand for 1 h at room temperature, and then holes (3 mm in diameter) were punched on the fibrin plate for sample application. To observe the Fibrinolytic activity, 100µl of sample solution was carefully dropped into each hole and incubated at $37\ ^0$C for 10 h. The activity of the Fibrinolytic enzyme was estimated by measuring the dimension of the clear zone on the fibrin plate and using standard streptokinase as a control [7].

RESULTS AND DISCUSSION

From the data of standard calibration curve, yield of preliminary batches was measured. Ageing period of strain, strain improvements by U.V. exposer were considered while designing preliminary batches. Total six batches were designed, from that batches N1 to N3 were of normal strain and M1 to M3 were of U.V. treated strain. Ageing period for the cultivation of strain were 2, 4, 7 days for N1.N2, N3 respectively. Same ageing period was taken for genetically improved strain. Enzyme concentration of preliminary batches was estimated by Lowry's method as discussed earlier. From the data of preliminary batches, it is concluded that U.V. treated strain produce higher amount of enzyme. Production of enzyme was decreased while increasing the incubation time from 2 days to 7 days. Batch M1 produced highest amount of enzyme among all six batches. So, on the basis of preliminary data, batch M1 was considered as a prominent batch and further study was carried out on it. Factorial experiment was applied on prominent batch to study the influence of media composition, inoculum volume and incubation time on the production yield of enzyme. Streptokinase yield of factorial design batches was described in (**Table 2**).

Regression analysis was carried out at 95%significant level. Regression analysis and data of Analysis of variance (ANOVA) were studied. From the statistical data, polynomial equation generated is as follows:

$$Y = -40.50 + 15.99X1 - 6.46X2 + 71.98X3 - 0.08X1X2X3$$

From regression analysis, R square is found to be 0.9933 which indicate that dependent and independent variables are significantly correlate with one another. Significance F value is found to be 0.00137 which is less than 0.05 which indicate that there are significance influence of variables on the response i.e. production yield The variable X1 has P value 0.0074 which is less than 0.05 as well as it has positive coefficient (b_1) which indicate that variable X1 is positively influenced on the response. The variable X2 has P value 0.045 which is also less than 0.05 as well as it has negative coefficient (b_2) which indicate that variable X2 is negatively influenced on the response. The variable X3 has P value 0.0055, less than 0.05 as well as has positive coefficient (b_3) which indicate that variable X3 is positively influenced on the response. Interacted variables have P value 0.37 which is greater than 0.05 which indicate that they have non-significant effect on the response.

To validate the generated equation of regression analysis, validation is carried out by taking one running batch according to process validation. Final yield of running batch was calculated by Lowry's protein estimation method. From the calculation yield was found to be 200.25 mg/ml. then predictable yield of enzyme by placing above values in polynomial equation. The final yield, calculated from equation was found to be 204.35 mg/ml which is complied with experimental yield. It means polynomial equation generated from regression data is validated for this experiment.

Overlay of two contour plot is made (**Fig 1**) by plotting two separate contour plot having incubation time constant in both of each. From the overlay of contour plot influence of three variables i.e. concentration of corn steep liquor, inoculum volume and incubation time on the final yield is studied.

Fibrinolytic activity was determined by the fibrin plate method. The results of different batches are in (**Fig 2**). Batch M1 gives highest activity among preliminary batches.

From the results of factorially design batches it was found that enzyme activity increases with concentration of the enzyme isolated. Factorial batch F6 gives highest activity among all factorial batches probably due to favorable condition for growing the mutated species and optimum biochemical process was occurred at that level due to change in genetic properties[7].

Radial Caseinolytic activity was performed on preliminary batches. From, preliminary batches it is found that enzyme isolated from U.V treated strain produce more activity than enzyme isolated from normal strain. Batch M1 gives highest activity among preliminary batches. Factorial batch F6 gives highest activity among all factorial batches.

Table 1: The amount of ingredients used in factorial batches

Ingredients	Factorial design Batches							
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**
Corn steep liquor (%)	8	8	8	8	12	12	12	12
Glucose (%)	7	7	7	7	7	7	7	7
$NaHCO_3$ (%)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
KH_2PO_4 (%)	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33
Inoculum volume (%)	8	8	15	15	8	8	15	15
Incubation time (Days)	2	3	2	3	2	3	2	3

Table 2: Real values of factorial design batches

Batch	X1	X2	X3	$X_1X_2X_3$	Yield (mg/ml)
F1	6	8	2	96	137.45
F2	6	8	3	144	207.33
F3	6	15	2	180	96.78
F4	6	15	3	270	145.42
F5	12	8	2	192	225.32
F6	**12**	**8**	**3**	**288**	**296.67**
F7	12	15	2	360	163.85
F8	12	15	3	540	227.45

Where, X1: Concentration of corn steep liquor (%) ; X2: Inoculum volume (%) ; X3: Ageing period (Days)

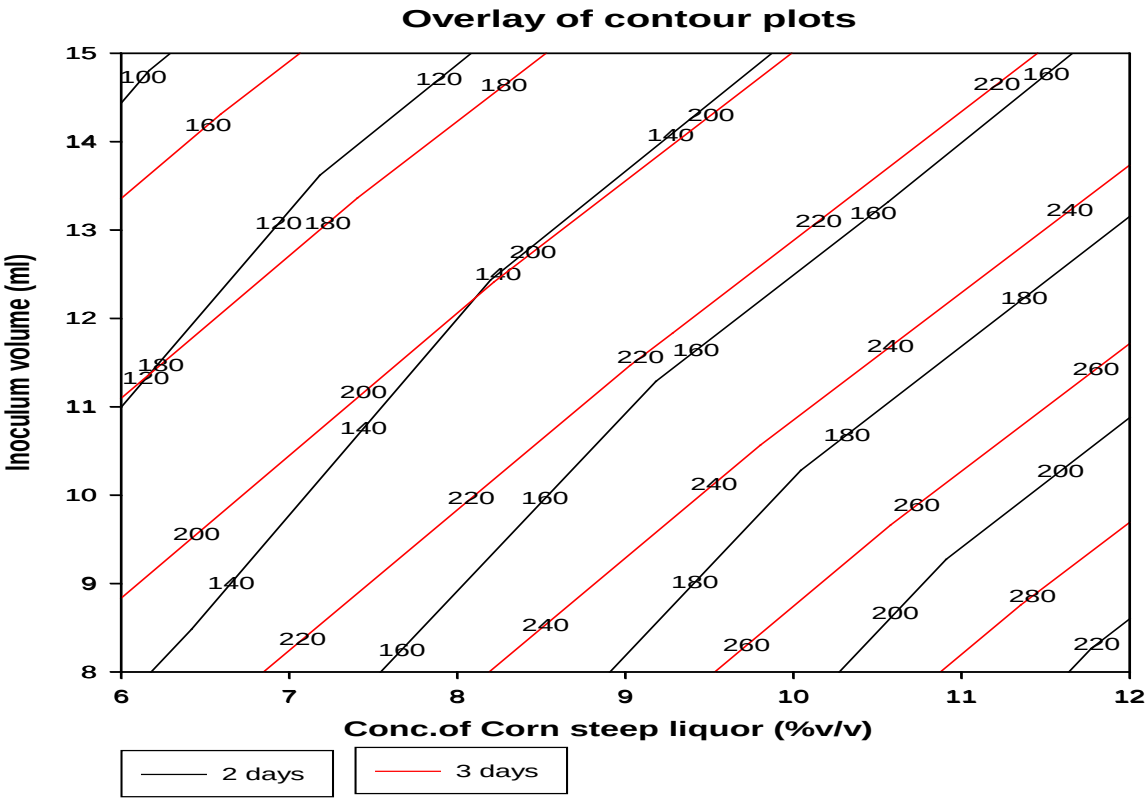

Fig 1: Overlay of contour plot for studying the internal parameters affecting on yield of streptokinase

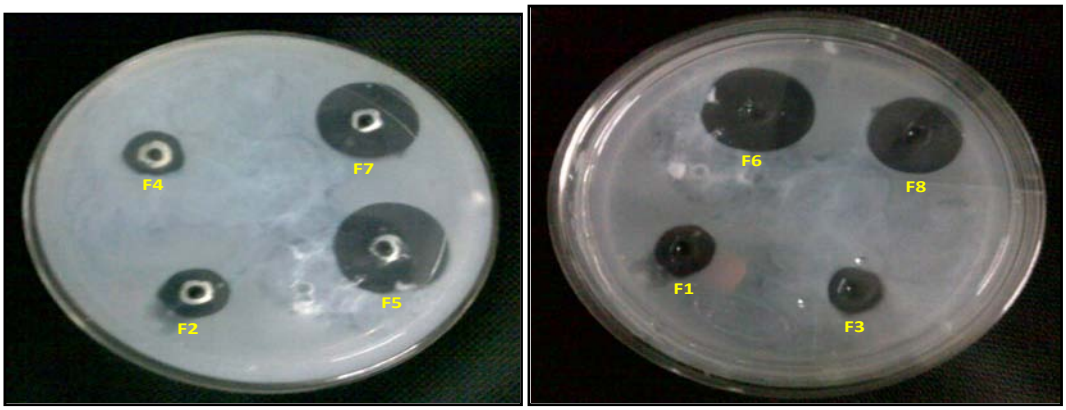

Fig 2: Zone of inhibition for Fibrinolytic activity of factorial design batches

Fig 3: Zone of inhibition of factorial design batches for Caseinolytic activity

CONCLUSION

From the above results, it can been concluded that the production yield of streptokinase enzyme by batch fermentation, as per stated in M1(45.04 mg/ml) batch was considered as the prominent batch as it produce five times higher yield than normal strain(9.68 mg/ml). Optimization of the same batch was carried out using 2^3 factorial designs indicated that F6 batch (296.67 mg/ml) was highest in production of streptokinase enzyme

almost six fold increments in production yield. From the regression analysis it was concluded that concentration of corn steep liquor and ageing period of strain are the parameters which have positive significant effect on enzyme yield while inoculum volume has negative significant effect on yield. All selected variables having a significant effect on final yield of enzyme (P value < 0.005). SDS PAGE analysis confirmed the presence of isolated streptokinase enzyme with comparison of standard streptokinase.

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