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MEDIA OPTIMIZATION FOR NATTOKINASE PRODUCTION FROM A MARINE ISOLATE AT A SHAKE FLASK LEVEL

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ABSTRACT

It wouldn't be an over statement to state that in today's world increased life expectancy is offered but not the quality of life. Among several diseases and disorders affecting the human population, Ischemic Heart Diseases (IHDs) and Cardiovascular Diseases (CVDs) pose a major threat globally attributing for more than 10 million and 200 million cases respectively per year. Both these diseases can be treated using enzyme drug, protease. Proteases are believed to act by dissolving the blood clot and thus enabling to restore the health condition among patients. Current treatment is based on an intravenous administration of streptokinase. Short half-life of the protein in biological fluids, demands increased drug dosage thereby increasing drug associated side effects. Nattokinase, another serine protease is currently being explored for its fibrinolytic potential. We, in our study have attempted to produce nattokinase from a marine isolate. The isolate when characterized for its microscopic and biochemical properties, exhibited its resemblance towards the Bacillus genus. This isolate was further subjected to upstream processing for nattokinase production. Effect of physical and nutritional parameters on enzyme production was evaluated by One Factor At a Time Approach (OFAT) followed by statistical optimization using Evolutionary Optimization Process (EVOP).

Keywords: Fermentation, Nattokinase, Fibrinolytic, CVDs, EVOP, etc.

I. INTRODUCTION

Today, Cardiovascular Diseases (CVDs) pose a major threat globally which has in turn decreased the life expectancy of humans. Cardiovascular disease is an abnormal function of the heart or blood vessels. It can cause an increase in risk for heart attack, heart failure, sudden death, stroke and cardiac rhythm problems, thus resulting in decreased quality of life and decreased life expectancy. The causes of cardiovascular disease range from structural defects, to infection, inflammation, environment and genetics. There are more than 200 million cases of CVDs per year. Number of CVD cases is growing at a rate of 25% per year. Most common CVDs like Coronary Heart Disease (CHD), cardiac arrest, stroke, high blood pressure, peripheral artery disease, congenital heart disease, congestive heart failure and arrhythmia are have of more than 20 million cases per year [1].

CVDs are treated with the help of protease enzymes. Enzymes are biocatalysts produced in human body from amino acids, which initiates and regulates countless biologic, metabolic and digestive reactions. Enzymes are catalytic and convert multiple target molecules to the desired products. Protease enzymes cleave the clot, blocking the blood vessels and thus relive the CVDs. This feature makes proteases as specific and potent drugs that can accomplish their utility as a therapeutic agent against the CVDs[2].

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Streptokinase is one of the serine protease enzyme currently used to treat the patients of CVDs. Streptokinase is intravenously administered into the body of the patient. The drawback of streptokinase is the short half-life which increases demand for increased dosage.Nattokinase is another serine protease enzyme. This nattokinase currently is being analyzed for its fibrinolytic potential. Nattokinase is robust and has relatively long half-life when compared to streptokinase. Nattokinase can be taken in tablet form by the CVD patients[3-4].

Generally, nattokinase is isolated from *Bacillus natto* or *Bacillus subtilis*[5]. We have attempted to produce nattokinase from marine isolate to check its pH and salt tolerance. Tolerance of nattokinase against pH and salt concentration will justify its stability in body fluids when it enters the human body. Enzyme production was maximized by optimization of fermentation media. Two stage approach of media optimization was employed in our study. Effect of certain critical parameters was evaluated by a traditional one factor at a time approach followed by a statistical approach EVOP.

II. MATERIALS AND METHODS

1.1 Materials

Glucose, Di -Potassium Hydrogen Phosphate, Magnesium Sulphate, Maltose, Calcium Chloride, Saffranin, Crystal Violet, Ethyl Alcohol, Starch, Methyl Red Reagent, Methyl Red Voges Proskauer (MR-VP), Mannitol, Tris Sodium Acetate, EDTA, Glycerol, Sodium Chloride, Potassium Chloride, Magnesium Chloride, Sodium Bicarbonate, Sodium Phosphate, TCA, Glacial Acetic Acid, Isopropanol, Chloroform, Isoamyl Alcohol, Bromophenol Blue, Anthrone were procured from Qualigens. From Himedia Labs Tryptone, Tyrosine, Agar-Agar Type I, Triple Sugar Iron, Ethidium Bromide, Kovac's Reagent Strip, Yeast Extract were procured. From Loba Chemicals Lugol's Iodine, Agarose, Tyrosine was procured. While, Soy Peptone, Sodium Dodecyl Sulphate were obtained from Merck and casein from SRL.

1.2 Microbial Cultures

The cultures used for nattokinase production such as standard *Bacillus natto*(NRRL B3666) and marine isolate (ICT-F1) were kindly gifted from Department of Food Engineering and Technology of Institute of Chemical Technology, Mumbai.

1.3 Maintenance of cultures

The bacterial cultures were preserved on 2% agar slants having the following composition in g/l: Glucose 10; yeast extract 10; $K_2HPO_4.3H_2O$ 1; $MgSO_4.7H_2O$ 0.5; pH 7.0 ±0.5[6].

1.4 Inoculum development

Standard strain *Bacillus natto* (NRRL B3666) was revived by transferring one loopful of organism from slant to the liquid medium consisting of (g/l) Glucose 10; yeast extract 10; $K_2HPO_4.3H_2O$ 1; $MgSO_4.7H_2O$ 0.5 and pH 7.0 ±0.5 in 100 ml conical flasks containing 25 ml of the above mentioned media [6].

Marine isolate (ICT-F1) was revived by transferring one loopfull of organism from slant to the liquid medium consisting of (g/l) Glucose 10; yeast extract 10; $K_2HPO_4.3H_2O$ 1; $MgSO_4.7H_2O$ 0.5 and pH 7.0 ±0.5 prepared using artificial sea water in 100 ml conical flask containing 25 ml of the above mentioned media [6].

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Artificial sea water had the following composition in g/lNaCl 24.7; KCl 0.66; $MgCl_2.6H_2O$ 4.7; $CaCl_2.2H_2O$ 1.9; $MgSO_4.7H_2O$ 6.3; $NaHCO_3$ 0.18.

1.5 Characterization of marine isolate

1.5.1 Morphology

Strains, NRRLB3666 and Marine- ICT-F1 were grown in petriplate on nutrient media agar by spread plate technique and incubated at 37°C for 24 h and its morphology with respect to shape, edge, margin, color, etc.was observed.

1.5.2 Biochemical Tests

The isolate was evaluated for its Gram's nature and an array of biochemical tests likeTriple sugar iron, Oxidase, Catalase, Starch hydrolysis, Citrate utilization, Methyl red, MR-VP, Indole, Mannitol and Acid and gas generation from glucose. All these tests were performed as mentioned in Bergey's Manual [7].Simultaneously the isolate was evaluated for its growth at increased temperature (50°C), anaerobic condition and higher salt concentration (5-10%).

1.6 Enzyme production

The media used for enzyme production had the composition in(g/l), Maltose 20; Soy peptone 10; Yeast extract 10;$K_2HPO_4.2H_2O$ 1; $MgSO_4.7H_2O$ 0.5; $CaCl_2.2H_2O$ 0.5, adjustedto pH 7.0 ±0.5 and was sterilized by autoclaving at 121ºC, 15 psig pressure for 20 minutes[8, 9]. 5% v/v of both the inoculums were then aseptically transferred into the previously sterilized production media dispensed into respectiveconical flasks for each strain and incubated at 37ºC, 180 rpmupto 72 h. Flasks were then withdrawn at an interval of 24 h and were analyzed for its media pH,dry cell weight (DCW), enzyme activity and protein concentration.After the particular incubation time, flasks were removed from incubator shaker and were centrifuged at 10ºC, 10,000 rpm for 20 minutes. The supernatant was then used for the determination of nattokinase activity and the pellet was discarded. Production media for marine isolate was prepared in artificial sea water while all other production parameters were maintained constant.

1.7 Batch Kinetic study of Nattokinase production

Batch study was performed using the same media as mentioned above. 5%v/v of both the inoculum were then aseptically transferred individually into the previously sterilized production media prepared and dispensed in respective conical flasks of each culture and incubated at 37ºC, 180 rpm upto 60 h. Flasks were withdrawn at an interval of 5 h and were analyzed for its media pH, dry cell weight (DCW), enzyme activity and protein concentration.After the particular incubation time, flasks were removed from incubatorshaker and were centrifuged at 10ºC, 10,000 rpm for 20 minutes. The supernatant was then used for the determination of nattokinase activity and the pellet was discarded.

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1.8 Evolutionary optimization of media for nattokinase production

Experimental design for studying the effect of three factors is given inTable1. Total of 10 experimental trials are divided into two blocks *viz*, I and II. Run E_{10} and E_{20} are referred to as control or center or mean values and the remaining runs have either higher or lower values than the center values. Values at the center level are based on our earlier investigations or can be obtained directly from the literature available. Experiment for each run was performed in duplicate and the average result and difference between the two results was calculated. Based on the average response, the individual and interaction effects and change in mean were calculated while the difference between the responses of two cycles was used in calculating the error limits. The design arrays as well as the calculations were done in accordance to formulae mentioned in[10].

In accordance with the above mentioned reference, EVOP design and the values used by us were as depicted in Table 1. Soy Peptone, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ were varied during optimization while all other experimental conditions were maintained constant. The harvesting time used for optimization was 40 h. The actual experimental EVOP design for trial I is as enumerated in Table 2.

Table 1: Concentration of variable components.

Components (g/50ml)	**Minus (-)**	**Zero (0)**	**Plus (+)**
Soy Peptone	0.15	0.25	0.35
$MgSO_4.7H_2O$	0.05	0.0625	0.075
$CaCl_2.2H_2O$	0.0075	0.0125	0.0175

Table 2: Experimental design of EVOP for threecomponents

Components(g/50ml)	**E_{10}**	**E_{11}**	**E_{12}**	**E_{13}**	**E_{14}**	**E_{20}**	**E_{21}**	**E_{22}**	**E_{23}**	**E_{24}**
Soy Peptone	0.25	0.15	0.15	0.35	0.35	0.25	0.35	0.15	0.35	0.15
$MgSO_4.7H_2O$	0.0625	0.05	0.075	0.05	0.075	0.0625	0.75	0.05	0.05	0.075
$CaCl_2 2H_2O$	0.0125	0.0075	0.0175	0.0175	0.0075	0.0125	0.0175	0.0175	0.0075	0.0075
Average Response	a_{10}	a_{11}	a_{12}	a_{13}	a_{14}	a_{20}	a_{21}	a_{22}	a_{23}	a_{24}

1.9 Analytical Methods

1.9.1 Enzyme assay

The enzymatic activity of nattokinase was determined by caseinolytic activity. One unit of caseinolytic activity (CU) was defined as the amount of enzyme releasing a unit of tyrosine equivalent per min per unit volume of enzyme, at pH 8 [11, 12].

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1.9.2 Protein concentration

The total protein concentration of the supernatant was estimated in accordance with the typical Folin Lowry method [13].

1.9.3 Sugar Estimation

Take 0.2 to 1ml of working standard solution of five different test tubes and add water to make up the volume to 1ml in each test tube. Add 4ml of Anthrone reagent and mix the contents well and keep it in boiling water bath for 10 min. Cool the test tubes to the room temperature and measure the optical density in colorimeter at 620nm. Simultaneously prepare a blank with 1ml of distilled water in 4ml of Anthrone reagent [14].

1.9.4 Dry cell weight determination

1 ml of microbial suspension was placed in a pre-weighed micro centrifuge tube and was centrifuged at 10,000rpm for 10 min. The supernatant was discarded and the tube was kept in an oven for 24h at 70ºC or till a constant weight was achieved.

II. RESULTS AND DISCUSSIONS

2.1 Morphology

Both the cultures were revived from their respective glycerol stocks in the sterilized nutrient broth media aseptically and incubated for 24 h under shaking condition of 180 rpm. Both the cultures were plated on sterilized nutrient agar plates by quadrant streak plate technique and incubated in incubator for 24 h at 35°C. Plates displayed the pure isolated colonies of both the cultures from which the morphological evaluation was made. The result is displayed in Table 3. The marine isolate ICT-F1 is a gram positive rod shaped bacteria.

Table 3: Morphological characterization

Sr No.	Characteristics	NRRL 3666	ICT-F1
1.	Color	White	White
2.	Shape	Rod	Rod
3.	Elevation	No	No
4.	Edges	Regular	Regular
5.	Transparency	Opaque	Opaque
6.	Grams nature	Grams +ve	Grams +ve

2.2. Biochemical tests

Each bacterial genera varies biochemically from other.Within one genera all the members exhibit similar biochemical characteristics. The identification of bacteria is a careful and systematic process that uses many different techniques to narrow down the types of bacteria that are present in an unknown bacterial culture.

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Multiple tests are performed to provide the fermentation abilities, presence of certain enzymes, and certain biochemical reactions. Qualitative observations were made on the tests, to identify the bacterial genus.

Various biochemical tests of the isolate ICT-F1 were done to identify the isolate according to themethods described in Bergey's Manual of Systematic Bacteriology(Prafulla, 2011). Table 4 summarizes the biochemical characteristics of isolate ICT-F1. The results showed typical characteristics of the *Bacillus*genus.

Table 4 : **Results of biochemical tests**

Sr. No	Biochemical Test	Result	Inference
1.	Triple sugar iron	+	Maltose has been hydrolyzed and then monomers have been fermented
2.	Oxidase	-	Absence of cytochrome oxidase
3.	Catalase	-	Absence of catalase
4.	Starch hydrolysis	+	Hydrolysis of starch
5.	Citrate utilization	-	No utilization of citrate
6.	Methyl red (MR)	+	Accumulation of end products
7.	Mannitol agar test	+	Utilization of mannitol
8.	Growth at 50ºC	-	No growth observed
9.	Indole test	-	Absence of tryptophanase enzyme
10.	MR-VP	-	No fermentation of glucose
11.	Acid and gas from glucose	-	No acid or gas production
12.	Growth in anaerobic agar	-	Culture is strictly aerobic

2.3. Production of nattokinase

The enzyme production from both the cultures was studied using the same media composition and environmental parameters at varied time intervals *viz*, 24 h, 48 h and 72 h. Magnitude of enzyme activity, protein concentration, DCW, pH and specific activity with respect to time for standard strain and marine isolate are given in Table 5.

Table 5: Enzyme production for standard *B. natto* and ICTF1against time.

Sr No	Time (h)	Enzyme Activity (U/l)		Total Protein Conc. (g/l)		Specific Activity (IU/g)		pH		DCW (g/l)	
-	-	*B. natto*	ICT-F1	*B. natto*	ICT-F1	*B. natto*	ICT-F1	*B. natto*	ICT-F1	*B. natto*	ICT-F1
1	0	0	0	0	0	0	0	7	7	0	0
2	24	13.145	5.634	1.478	1.777	8.896	3.171	7.5	6.01	3.9	0.6
3	48	40.845	10.25	2.835	2.414	14.409	4.246	6.8	5.69	4.7	2.6
4	72	15.252	1.847	2.443	2.477	6.244	0.746	6.6	6.28	2.9	1.5

As seen in the above Table 5, the enzyme activity in standard strain was obtained maximum at 48 hi.e. 40.845±0.005 U/l and specific activity of 14.409 IU/g with biomass concentration of 4.7g/l. Similarly the enzyme activity in marine isolate was obtained maximum at 48h i.e. 10.25±0.003 U/l and specific activity of 4.246 U/g with biomass concentration of 2.6g/l. It was observed that the enzyme activity of standard strain NRRL B 3666 was 4 folds higher than marine strain ICT-F1. The pH of both the strains was seen to be decreasing with increase in time. This may be because of utilization of sugar by organism for their growth, as the time increases various organic acids are secreted in batch through metabolic pathways hence resulting in decreased media pH [15, 16].

2.4. Standard batch kinetics

Enzyme production and microbial growth profile for *B. natto* was studied at a time interval of 5 h till 60 h. Batch kinetics was performed with the aim to produce and check nattokinase production as well as the microbial growth pattern in batch mode. The enzyme activity was obtained highest at 50 h of 57.042±0.01353 U/l with protein concentration of 1.174 g/l and biomass concentration of 4.2 g/l. It was seen that enzyme activity goes on decreasing above 50 h while protein concentration was increasing. This could be because of the fact that the organism might be secreting other byproducts which may be other proteins. Also values of enzyme activity shows that there is no much utilization of sugar above 50 h and the sugar may act as catabolic suppresser for enzyme production. The organism utilizes nitrogen source (soy peptone) for the production of nattokinase enzyme which produces ammonia in the batch media and led to an increase in the pH i.e. towards basic during the fermentation course time.

2.5. Marine batch kinetics

Results of batch kinetics of marine isolate ICT-F1 are depicted below in Table 5.6 and Figure 5.5. Marine isolate was found to secrete the maximal amount of enzyme at 40 h itself as compared to the standard strain with 50 h of batch time. The enzyme activity was obtained highest at 40 h of 26.839±0.009 U/l with protein concentration of 1.423 g/l and biomass concentration of 4.12 g/l. It was seen that enzyme activity goes on decreasing above 40 h while protein concentration was increasing. This can be because of fact that the organism might be secreting other byproducts which may be other proteins. Also values of enzyme activity shows that there is no much utilization of sugar above 40 h and the sugar may act as catabolic suppresser for enzyme production. The variation in the pH was obtained very less.

The overall trend in the batch data for both the cultures was found to be almost similar except the fermentation time and magnitudes of the metabolites.

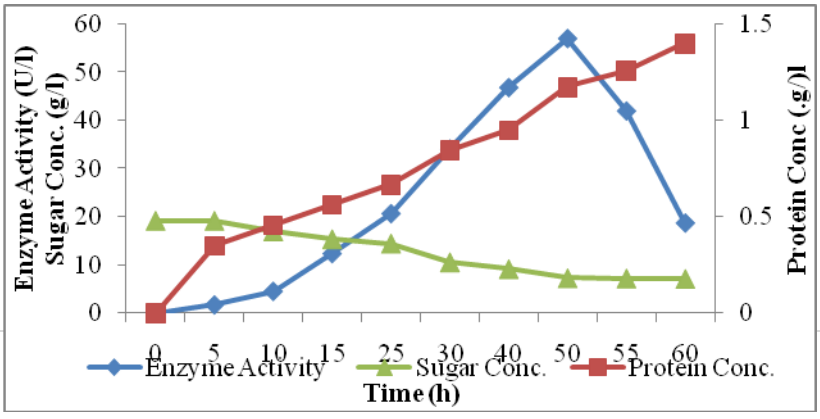

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Figure 1: Batch kinetics for *B. natto*

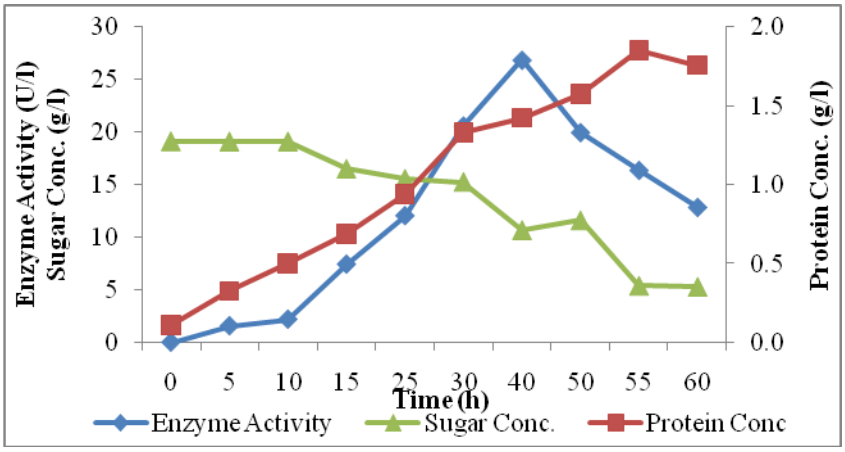

Figure 2: Batch kinetics of ICT-F1.

2.6. EVOP

Keeping the basal media composition and all other factors constant as mentioned in section 4.7 and only varying the three parameters which were soy peptone, calcium chloride and magnesium sulfate at three different levels as enumerated in Table 5.7, the enzyme production was studied at varied runs with an aim to optimize the fermentative media for enzyme production. The batch was harvested after 40 h by centrifuging at 10 °C, 10000 rpm for 20 minutes after which supernatant was tested for enzyme titre. The experiment as suggested by authors was performed in duplicates and its average and difference values were calculated. The results are given below in Table 6 and effects and error limits are given in Table 7. From the data analysis it can be concluded that the change in mean is negative and large as compared to the error limits suggests that the magnitudes of the three variables that we have decided are valid and can be considered for optimization study. However the individual and interaction effects are large as compared to the error limits of the effects (0.494) that suggest that the true optimum conditions are yet to attain. As per the rules of decision making of EVOP, soy peptone (2.787) and calcium chloride (3.140) have a higher positive effect indicating that they exhibit the positive correlation towards the enzyme production and for the next EVOP cycle their values needs to be increased. In case of magnesium sulfate there exists a negative correlation between its concentration and enzyme titre of -5.605 and in the next cycle its concentration needs to be lowered than used in current experimental cycle. Thus to optimize the media for nattokinase production another trial of EVOP cycle needs to be undertaken by considering the results of the first cycle [17, 18, 19].

Table 6: Results of EVOP design.

Factor	E10	E11	E12	E13	E14	E20	E21	E22	E23	E24
SOY PEPTONE	0.25	0.15	0.15	0.35	0.35	0.25	0.35	0.15	0.35	0.15
MgSO4	0.0625	0.05	0.075	0.05	0.075	0.0625	0.75	0.05	0.05	0.075
CaCl2	0.0125	0.0075	0.0175	0.0175	0.0075	0.0125	0.0175	0.0175	0.0075	0.0075
EA 1 (U/l)	39.671	22.066	15.962	21.596	11.737	35.446	29.812	25.587	30.047	16.667
EA 2 (U/l)	38.028	21.127	16.667	19.953	13.85	33.333	29.577	27.465	28.638	17.37
AVG EA (U/l)	38.850	21.597	16.315	20.775	12.794	34.390	29.695	26.526	29.343	17.019

Difference	1.643	0.939	-0.705	1.643	-2.113	2.113	0.235	-1.878	1.409	-0.703

Table 7:Calculation of effects.

Effect of	**Calculation of effects**
Soy peptone	2.787
$MgSO_4.7H_2O$	-5.605
$CaCl_2.2H_2O$	3.140
Effect of soy peptone*$MgSO_4.7H_2O$	1.790
Effect of $MgSO_4.7H_2O$* $CaCl_2.2H_2O$	1.027
Effect of $MgSO_4.7H_2O$* $CaCl_2.2H_2O$	4.959
Effect of soy peptone* $MgSO_4.7H_2O$* $CaCl_2.2H_2O$	7.776
Change in mean effect	-11.890
Sigma (σ)	0.492
σ_1	0.422
σ_2	0.563
F	0.3
Error limits	
For average	± 0.696
For effects	± 0.494
For change in mean	± 0.439

III. CONCLUSIONS

- Marine isolate ICTF1 is a gram negative rod shaped bacilli belonging to genus *Bacillus*
- Standard culture *Bacillus subtilis* NRRL B3666 produced maximum amount of nattokinase (40.845 ± 0.005 U/l) as compared to the marine isolate ICTF1 (10.25 ± 0.003 U/l) in the media suggested by Liu et al., (2004) at 48 h of fermentation time.
- Batch kinetic data of *Bacillus subtilis* NRRL B3666 secreted maximal amount of the enzyme (57.042 U/l) at 50 h of fermentation time. The enzyme is secreted during towards the end of logarithmic phase of bacterial growth. Similarly, the batch kinetic data of marine isolate ICT-F1 secreted maximal amount of enzyme (26.839 U/l) at 40 h of fermentation time in the logarithmic growth phase of bacteria. Although the marine isolate secreted low enzyme than the standard culture but a significant reduction in batch time by 10 h is observed. This is an encouraging result for industrial perspective.
- Statistical media optimization using evolutionary methodology approach was attempted by us for ICTF1. From the magnitude of effects of parameters it can be concluded that the experimental design of EVOP cycle I was valid but we could not obtain the optimized media composition. The enzyme activity was found to increase from 26.839 U/l in batch data to 38.850 U/l in EVOP cycle I indicating a meager increase in enzyme activity

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