Optimization of recombinant Cellulase (Cel A) production in E. coli using statistical modelling

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Abstract— There are various methods of optimizing the fermentation medium and fermentation process conditions like factorial design, biological mimicry, Evolutionary operation factorial design, Plackett-Burman design, Central composite design and Response surface methodology. Each method has advantages and disadvantages. In our study, we have chosen Plackett-Burman design, Central composite design and Response surface methodology to optimize the fermentation medium and fermentation process conditions for Cellulase (CelA) production and purification. The modified HCD media has given good Cellulase (CelA) expression (2.8g/L) and solubility (1.9 g/L) and enzyme activity of 7.9 IU/ml was achieved.

Key words: CelA, E. coli, Plackett-Burman design

I. INTRODUCTION

Cellulase (CelA) refers to a group of enzymes which, acting together, hydrolyze cellulose. Cellulose is a linear polysaccharide of glucose residues connected by β-1,4 linkages. Native crystalline cellulose is insoluble and occurs as fibers of densely packed, hydrogen bonded, anhydroglucose chains of 15 to 10,000 glucose units. Its density and complexity make it very resistant to hydrolysis without preliminary chemical or mechanical degradation or swelling. Cellulose is the most abundant organic source of food, fuel and chemicals. However, its usefulness is dependent upon its hydrolysis to glucose. Acid and high temperature degradation are unsatisfactory in that the resulting sugars are decomposed; enzymatic degradation Cellulase A (CelA) is the most effective means of degrading cellulose into useful components. Although Cellulase (CelA)s are distributed throughout the biosphere, they are most prevalent in fungal and microbial sources.

The massive usage of petroleum and petroleum products in the last decade, with the consequent reverse effect on minimizing consumption of these unsustainable resources, has increased the demand for the development of renewable sources [1,2]. Currently, based on the carbon neutrality concept, two sources of biofuels have entered the marketplace; ethanol from cellulosic materials and biodiesel from soybean or palm oil [3]. The bioconversion of lignocellulosic materials is a challenging process which requires two steps. During the bioconversion process, the lignin and the hemicellulosic parts are first degraded into simpler sugars and/or organic acids, followed by a deoxygenating step to produce a liquid fuel [4]. Design of a genetically modified microorganism for direct lignocellulosic biomass conversion purposes has recently been taken into consideration [5]. The production of several types of fuel through direct lignocellulosic biomass conversions has been demonstrated by various studies [6,7]. A genetically engineered E. coli capable of degrading pectin-rich lignocellulosic biomass by cellulolytic and pectinolytic activities has been developed [8]. E. coli has been considered a convenient biocatalyst in biofuel production for its fermentation of glucose into a wide range of short-chain alcohols [9,10], and production of highly deoxygenated hydrocarbon through fatty acid metabolism [11,12]. Moreover, the ability to ferment several pentoses and hexoses makes E. coli an ideal ethanologen for biofuel production [5,13]. The aim of this study was to optimize the CelA (from Neocallimastix spp.) expression in E. coli cells by Plackett-Burman and RSM methods and subsequently, purification and enzymatic activity using CMC substrate.

II. MATERIALS AND METHODS

A. Production of recombinant Cellulase (CelA) in E.coliBL21DE3-pRSETA-CelA using modified HCD media:

The recombinant Cellulase (CelA) production was tested with three media, namely Terrific Broth Glycerol, Terrific Broth Glucose and Modified High Cell Density (mHCD).

Components	g/L
Di Ammonium hydrogen phosphate	4
Potassium Di hydrogen phosphate	13
Citric acid anhydrous	1.7
Thiamine hydrochloride	0.1
Yeast extract	3
Magnesium sulphate heptahydrate	1.2
Dextrose anhydrous	10
Trace metal solution*	10 mL/L
Calcium chloride Di hydrate	0.0002
EDTA sodium salt	0.000084
Cobaltous chloride hexahydrate	0.000025
Manganese chloride tetrahydrate	0.00015
Coppersulphate pentahydrate	0.00002
Boric acid	0.00003
Sodium molybdate dehydrate	0.000025
Zinc sulphate heptahydrate	0.00017
Ferrous sulphate heptahydrate	0.001
Ammonium sulphate	0.001

Table 1: Modified HCDF medium composition (reference)

B. Fermentation conditions:

E.coli BL21 DE3 culture were inoculated in three different culture media- terrific broth with glucose, terrific broth with glycerol with 1% (v/v) and modified HCDF medium. Seed was used as inoculum culture into 1liter baffled flasks containing 250ml of the sterilized three different medium. The flasks were incubated at 37°C in an incubator shaker, agitated at 200 rpm for 2-5hours. Then cultures were reached OD600 1.5-2.0 induced with 1.0 mM IPTG solution. Then allow the induction duration for 4-6 hrs, after that harvest and

centrifuged for 25 minutes at 8000rpm, temperature 4-10°C. Samples were withdrawn at before and after induction period at different time intervals for expression analysis. The medium showing maximum enzyme production was selected for further optimization of different production parameters. Three different medium TB with glucose, TB with glycerol and modified HCDF and were tested for the growth of E.coli and expression of recombinant Cellulase (CelA), in this the better medium was selected as the best of three medium, best single strain was selected from the 4 different host systems and further optimization was done using Plackett - Burman and CCD RSM statistical methods.

Use of expression of recombinant Cellulase (CelA) in E.coli by recombinant process require its production in high amount at low cost and hence current focus on research is oriented towards the optimization of fermentation medium and process variables by statistical methods. A numeral statistical experimental design with Plackett - Burman and response surface methodology (RSM) has been employed for optimizing recombinant Cellulase (CelA) production from recombinant E.coli cells. It is a well-known fact that expression of Cellulase (CelA) and its activity in E.coli by recombinant process is greatly influenced by media components and process variables. The experimental designs were applied in two stages, first to identify the significant nutrients and culture conditions for recombinant Cellulase (CelA) production using Plackett - Burman design criteria. Subsequently, the significant variables resulted from Plackett - Burman design was optimized by response surface methodology.

C. Screening of significant variables by Plackett-Burman design:

The purpose of this optimization step is to identify which variables of the process have significant effect on recombinant Cellulase (CelA) production from recombinant E.coli cells. Plackett - Burman experimental design was used for initial screening of the important medium components for the strain of recombinant E.coli cells with respect to their main effect on r-cellualse production. Seven numerical variables Di Ammonium hydrogen phosphate (NH4)2HPO4, potassium di hydrogen phosphate, Yeast extract, Dextrose anhydrous , Inoculum size, Temperature and pH were selected and a 12 run Plackett - Burman design at 2 levels was conducted (Table No.2), with the concentration at +1 and –1 (Table No.1).

This design does not consider the interaction effects between the variables and is used to screen the important variables affecting recombinant Cellulase (CelA) production [14]. It can be represented by first-order polynomial equation: $Y= \beta 0+ \Sigma \beta i\ xi$

Where Y represents the response, β0 is the model coefficient, βi is the linear coefficient and xi is the level of the independent variable.

Factor	Level (g/L or %)		
	-1	0	+1
Di ammonium hydrogen phosphate	3	3.5	4
Potassium di hydrogen phosphate	11	12	13
Yeast extract	3	4	5
Dextrose anhydrous	10	12.5	15
Inoculum size (%)	2.5	5	7.5
Temperature	36	37	38
pH	6.8	7.0	7.2

Table 1: Tested independent variables and their levels in the Plackett-Burman experiment

Factors: 7 Replicates: 1
Base runs: 12 Total runs: 12
Base blocks: 1 Total blocks: 1

RunOrder	Potassium di hydrogen phosphate (G/L)	Di Ammonium hydrogen phosphate (G/L)	Yeast extract (G/L)	Dextrose anhydrous (G/L)	Inoculum size (%)	Temperature (°C)	pH
	A	B	C	D	E	F	G
1	13 (+)	3(-)	5(+)	10(-)	2.5(-)	36(-)	7.
2	13(+)	4(+)	3(-)	15(+)	2.5(-)	36(-)	6.
3	11(-)	4(+)	5(+)	10(-)	5(+)	36(-)	6.
4	13(+)	3(-)	5(+)	15(+)	2.5(-)	38(+)	6.
5	13(+)	4(+)	3(-)	15(+)	5(+)	36(-)	7.
6	13(+)	4(+)	5(+)	10(-)	5(+)	38(+)	6.
7	11(-)	4(+)	5(+)	15(+)	2.5(-)	38(+)	7.
8	11(-)	3(-)	5(+)	15(+)	5(+)	36(-)	7.
9	11(-)	3(-)	3(-)	15(+)	5(+)	38(+)	6.
10	13(+)	3(-)	3(-)	10(-)	5(+)	38(+)	7.
11	11(-)	4(+)	3(-)	10(-)	2.5(-)	38(+)	7.
12	11(-)	3(-)	3(-)	10(-)	2.5(-)	36(-)	6.

Table 2: Plackett-Burman Design

D. Optimization by Central Composite Design (CCD):

After screening for the significant variables by the PBD method, the statistically-based optimization using central composite design (CCD) was carried out under response surface methodology (RSM), which is widely used in experiments where the main interest is in modelling the relationship between a number of quantitative factors and one or more response variables, and locating the combination of the factor levels that yields the best response [15]. The benefits from this method were the decreasing number of experiments, and in using time and material resources. Furthermore, the analysis performed on the results is easily realized, and experimental errors are minimized [16]. A CCD consists of a 2n factorial augmented by 2n axial points and nc center points that are explained through the following equation:

$A= 2n + 2n + Co$

Where A is the total number of experiments, n number of variables that are used and C_o is the number of center points [17]. The corresponding factors are coded according to the following equation

$$X_{coded} = \frac{[X_{act} - X_{cen}]}{\left[\frac{X_{cen} - X_{min}}{\sqrt{n}}\right]}$$

The second-order polynomial equation that describes the relationships between the factors and the dependent variable and gives information about interaction between variables (factors) in their relation to the dependent variable is:

$$Y = \beta_o + \sum_{i-1}^{n} \beta_i X_i + \sum_{i-1}^{n} \beta_{ii} X_i^2 + \sum_{i-1}^{n} \sum_{j-1}^{n} \beta_{ij} X_{ij}$$

Where Y is the predicted response, X_i to X_n the input variables, β_0 the intercept term, β_i the linear effects, β_{ii} the squared effects, and the β_{ij} interaction term.

In this study, five factors Yeast Extract, Glucose, IPTG, Inoculum size and Induction Temperature were chosen as independent variables as shown in Table 3, Enzyme

activity U/mL were chosen as dependant output variable. Thirty two experiments based on the CCD were carried out with different combinations of variables and presented in table.4. The "Minitab17" Software was used for the regression analysis and graphical analysis of the data obtained.

Factor	Variable code	Level		
		-1	0	+1
Yeast	A	3	4	5
Inoculum Size	B	2.5	3.75	5
IPTG (mM)	C	0.250	0.375	0.5
Induction Temp	D	24	25	26
Dextrose (g/L)	E	4	6	8

Table 3: Codes and actual levels of the independent variables for design of experiment using CCD

Factors: 5 Replicates: 1
Base runs: 32 Total runs: 32
Base blocks: 1 Total blocks: 1

Two-level factorial: Half fraction
Cube points: 16
Center points in cube: 6
Axial points: 10
Center points in axial: 0
α: 2

Run Order	Yeast Extract (g/L)	Inoculum Size (%)	IPTG (mM)	Induction Temperature(°C)	Dextrose (g/L)
	A	B	C	D	E
1	3 (-1)	2.5(-1)	0.25(-1)	24(-1)	8(1)
2	5(1)	2.5(-1)	0.25(-1)	24(-1)	4(-1)
3	3(-1)	5(1)	0.25(-1)	24(-1)	4(-1)
4	5(1)	5(1)	0.25(-1)	24(-1)	8(1)
5	3(-1)	2.5(-1)	0.5(1)	24(-1)	4(-1)
6	5(1)	2.5(-1)	0.5(1)	24(-1)	8(1)
7	3(-1)	5(1)	0.5(1)	24(-1)	8(1)
8	5(1)	5(1)	0.5(1)	24(-1)	4(-1)
9	3(-1)	2.5(-1)	0.25(-1)	26(1)	4(-1)
10	5(1)	2.5(-1)	0.25(-1)	26(1)	8(1)
11	3(-1)	5(1)	0.25(-1)	26(1)	8(1)
12	5(1)	5(1)	0.25(-1)	26(1)	4(-1)
13	3(-1)	2.5(-1)	0.5(1)	26(1)	8(1)
14	5(1)	2.5(-1)	0.5(1)	26(1)	4(-1)
15	3(-1)	5(1)	0.5(1)	26(1)	4(-1)
16	5(1)	5(1)	0.5(1)	26(1)	8(1)
17	2(-2)	3.75(0)	0.375(0)	25(0)	6(0)
18	6(2)	3.75(0)	0.375(0)	25(0)	6(0)
19	4(0)	1.25(-2)	0.375(0)	25(0)	6(0)
20	4(0)	6.25(2)	0.375(0)	25(0)	6(0)
21	4(0)	3.75(0)	0.125(-2)	25(0)	6(0)
22	4(0)	3.75(0)	0.625(2)	25(0)	6(0)
23	4(0)	3.75(0)	0.375(0)	23(-2)	6(0)
24	4(0)	3.75(0)	0.375(0)	27(2)	6(0)
25	4(0)	3.75(0)	0.375(0)	25(0)	2(-2)
26	4(0)	3.75(0)	0.375(0)	25(0)	10(2)
27	4(0)	3.75(0)	0.375(0)	25(0)	6(0)
28	4(0)	3.75(0)	0.375(0)	25(0)	6(0)
29	4(0)	3.75(0)	0.375(0)	25(0)	6(0)
30	4(0)	3.75(0)	0.375(0)	25(0)	6(0)
31	4(0)	3.75(0)	0.375(0)	25(0)	6(0)
32	4(0)	3.75(0)	0.375(0)	25(0)	6(0)

Table 4: CCD design table

E. Extraction and Purification of recombinant Cellulase A (Cel A):

E.coli was cultured in submerged conditions starts with the factors at 36.5°C, pH 7.0 with 200 rpm with all other optimized parameters. After 22 hours of submerged fermentation bulk crude enzyme extract was produced that was utilized for purification. Following steps were carried out for purification of recombinant Cellulase A (Cel A).

F. Separation of Biomass from Fermentation Broth:

After 4-5 hrs of induction, the harvest fermented culture sample was used to separate the cell biomass by centrifugation at 8,500 rpm for 15 minutes at 4 °C. Supernatant was discarded and the cell pellet was kept in a deep freeze at -20°C used for further purification steps described below.

G. Homogenization of cell biomass:

The culture was pelleted by centrifugation, then that cell pellet was taken and wash given twice by washing buffer to remove the fermentor medium components. Then the washed cell pellet was suspended by adding of lysis buffer on the basis of 10mL per 1gram of cell- biomass. Then cell lysate in native conditions was prepared by lyse the culture using homogenizer at 11000 psi for 3No's of cycle where the > 80% cell lysis taking place and the lysate sample centrifuged to remove debris and the lysate supernatant contains soluble protein fraction was purified by Ni-NTA agarose column.

H. TFF:

Tangential flow filtration (TFF) is a rapid and efficient method for primary separation and purification of biomolecules. Before loading on Ni-NTA agarose column, the lysate sample is to be free from cell debris because of increasing the binding efficiency by separating unwanted bio molecules. This can be achieved by the method of using TFF (Tangential flow filtration) by passing through 0.45µ TFF cassettes to get the clear supernatant permeate is subjected to load Ni-NTA column chromatography. 1.1.3

I. Affinity column chromatography by using Ni-NTA matrix:

The permeate (Approx.1500 ml) of TFF was subjected to Ni-NTA purification by adding 150 mL of Ni-NTA resin (100 mL resin has a capacity to bind 400–800 mg 6xHis-tagged protein) in a GE - XK 16 column and connected to the FPLC (akta system). The XK-16 column with Ni-NTA resin was equilibrated with equilibration buffer. Further purification of centrifuged cell lysate was done by passing through the above mentioned column. After passing the cell lysate through Ni-NTA column and followed by washing with wash buffer twice the column volumes (CVs); The column was then eluted with elution buffer of three times of CVs. All the eluents were subjected to SDS-PAGE analysis for the determination of fractions with over expressed recombinant Cellulase (CelA) for protein concentration by SDS-PAGE densitometry.

J. Zymogram:

The activity of the purified enzyme was determined by zymogram analysis on polyacrylamide gel electrophoresis (PAGE) using 10% separating gel and 4% stacking polyacrylamide gels without mercaptoethanol, according to standard protocol (Laemmli, 1970). The separating gels were incorporated with either 1% CMC for CMCase assay analysis. To detect the cellulolytic activity of E.coli expressed protein, we used zymograms (Schwarz et al., 1987), as described below. This substrate was added before

polymerization to the resolving portion of 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

To prevent aggregation, CMC was added slowly to the gel mixture while stirring. Gel polymerization was induced after all CMC was dissolved. Gels were allowed to polymerize overnight at room temperature, then kept at 4 °C until used. the column purified samples solubilized in 1 volume of sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue) (Laemmli, 1970). Solubilized samples (100 µg of protein) were heated at 70 °C for 20 min to partially denature enzymes and reduce smearing of activity due to continuous enzymatic activity during electrophoresis (data not shown). Following heating, samples were briefly centrifuged to collect evaporated solution, and loaded in duplicate on gels for subsequent detection of total protein and Cellulase (CelA) activity.

Electrophoretic separation was carried out at 4 °C at constant voltage (100 V) for approximately 4 h, or until the sample buffer dye reached the bottom of the gel. For cellulolytic activity staining, gels were washed five times (6 min each) in 50 ml of wash buffer (0.1 sodium succinate pH 5.8 plus 10 mM DTT). Gels were incubated for 30 min at 60 °C in wash buffer without DTT to develop Cellulase (CelA) activity by incubation in a 0.1% Congo Red (Acros Organics) solution for 10–15 min at room temperature. Gels were destained using 1 M NaCl until clear activity bands were visible. For increased visualization of activity bands, glacial acetic acid was added (2% v/v) to the NaCl solution (Waeonukul et al., 2007). Following acetic acid treatment, gels turned dark-purple in color with activity bands remaining as clear zones. Images of gels were acquired using a Gel Doc.

K. Cellulase A (Cel A) Assay:

1ml of enzyme was incubated with 10mg carboxymethylcellulose (CMC) or 10 mg of Whatman No.1 Filter paper (FP) for the determination of carboxymethylCellulase (CelA) (CMCase) and filterpaperase (FPase) activities, respectively, with 4ml of 0.1M acetate buffer (pH 6.0). The incubations were carried out for 30 minutes and 1 hour (at 60oC) for CMCase and FPase activities. After incubation, the reaction was stopped by immediate cooling in an ice bath and the reducing sugars were determined by DNS method (Miller, 1959). One unit of CMCase activity is equivalent to 1 M of reducing sugar (glucose equivalents) released per minute with glucose as standard. One unit of FPase activity is equivalent to 1 M of reducing sugar (glucose equivalents) released per hour with glucose as standard under assay conditions described above. The Cellulase (CelA) activities were estimated on crude and column purified samples. One unit of Enzyme activity is defined as the amount of enzyme releasing 1µmol of glucose produced per minute under standard assay conditions. Enzyme units (U/ ml) were calculated by the following formula

$U/ml = (X/T) \times 2$

Where X is the µmol of glucose produced by 0.5ml enzyme sample from the substrate, T is the assay time in minutes.

Glucose standard curve: A 3000 µmol Glucose stock solution was prepared which was serially diluted and pipette into a test tube with final strengths ranging from 300, 600, 900, 1200, 1500, 1800, 2100, 2400, 2700, and 3000 µmol/1ml of each dilution was analyzed for glucose content.3ml of each dilution was added to each tube and boiled in a water bath for 5 minutes. After boiling the tubes were transferred to cool water bath and the color thus developed was read at 540 nm. Optical densities of each dilution were plotted against their corresponding dilutions using Microsoft Office Excel software. The concentrations of glucose in the unknown sample were calculated by the formula.

$Y = 0.0006X - 0.1979$

$X = (Y+0.1979)$

0.0006

III. Results and Discussion

A. Production of recombinant Cellulase (CelA) in E.coliBL21DE3-pRSETA-CelA using modified HCD media:

The effect of using three different types of medium TB glycerol, TB Glucose and mHCD (modified HCD) on growth of E.coli BL21 DE3-pRSETA-cel1A and production of recombinant Cellulase (CelA) (recombinant Cellulase (CelA)) is shown in table -5. The highest growth of E.coli BL21 DE3-pRSETA-cel A (1.9 to 4.3 OD600, Fig. - 1) and highest enzyme activity of cel A (recombinant Cellulase A (CelA) (2.1 to 5.2 U/mL, Fig.-1) was obtained in at 250ml shake flask level. A great reduction in the production and activity of Cellulase (CelA) A was observed in fermentation using TB Glycerol though growth was slightly inhibited. The growth, production and activity were greatly inhibited in shake flask studies using TB Glucose medium. The final enzyme concentration obtained in fermentation using modified HCDF medium (1.35-1.55mg/mL) was 1.5 fold higher than those obtained in TB Glycerol (0.95-1.15mg/mL) and TB Glucose (0.85-0.97mg/mL) respectively. Before any other considerations, it can be seen from Table 5, Fig. -1, that the production of recombinant Cellulase A (CelA) was associated with the bacterial growth as well as induction with the IPTG at the concentration of 0.25mM.

The relationship between biomass and recombinant Cellulase A(CelA) production is extremely important in large-scale production. Subsequently, in order to lower the costs of production and the purification process of the recombinant Cellulase (CelA) obtained, it is essential to pay attention to two criteria:

Attaining the maximum yield of recombinant Cellulase A (CelA) at the end of the cultivation in the bioreactor.

This value is compatible with the results obtained in the current study (Table-5). Furthermore, the authors concluded that by operating with constant aeration and agitation rates, they could obtain an increase in Cellulase A (Cel A) production. Because of this study, they suggested that Cellulase A (Cel A) production is related to cellular growth, which is constrained by the limited availability of oxygen.

The results which are from this experiment, where the modified HCD medium was used (average of 1.30-1.5mg/mL) shows that this medium can be considered an interesting possibility for Cellulase A (celA) production, since not only does it give reasonable final yield values, it is a semi synthetic medium, that means, it is chemically defined except yeast extract, whereas the other two media used in this study are chemically undefined. The selected modified HCD

medium was further optimised using Placket-Burman and Response surface methodology designs.

S No	Description	Fermentation medium					
		TB Glycerol		TB Glucose		mHCDF	
		OD_{600}	U/mL	OD_{600}	U/mL	OD_{600}	U/mL
1	*E.coli BL21 DE3*	2.7	3.3	1.9	2.1	4.1	5.2

Table 5: Effect of three medium TB Glycerol, TB Glucose and modified HCD on growth of E.coli and production of Cel A

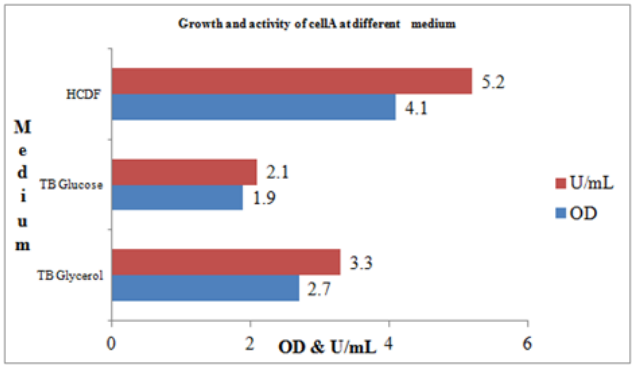

Fig. 1: Growth and Activity of Cel A TB Glycerol, TB Glucose and mHCD

B. Screening of significant variables by Plackett-Burman design:

The screening of fermentation media and process variables of their significance was carried out using Plackett-Burman design. The effect of seven variables namely Di ammonium hydrogen phosphate, potassium di hydrogen phosphate, yeast extract, Dextrose anhydrous, Initial pH, Inoculum size and Temperature on growth and production of recombinant Cellulase (CelA) by submerged fermentation by E.coli BL21 DE3 were analysed. (Table- 6) shows the Plackett – Burman experimental design of experiments and the results obtained from the experiments which are generated by the MINITAB 17 software. From the table No. 6, it was observed that the variation in recombinant Cellulase (CelA) expression was 1.25 – 1.73g/L, the activity was 3.8 to 6.1 U/mL and the growth in OD600 was 1.65 to 4.9 in batch mode. The two values of each variable {maximum (+) and minimum (-)} were chosen such that the difference between the two values (+ and -) is large enough to ensure that it includes the peak area for the maximum Cellulase A (CelA) production. The maximum and minimum values of seven variables are given in (Table-1)

On analysis of regression coefficient (t-value) of seven variables Inoculum size, and Yeast extract has shown positive effect on growth and recombinant Cellulase (CelA) A production, whereas potassium di hydrogen phosphate, Di ammonium hydrogen phosphate, pH ,Dextrose an hydrous and Temperature has shown negative effect on growth as well as recombinant Cellulase A (CelA) production (Table 6). There is a close agreement between the experimental values of recombinant Cellulase (CelA) production and theoretical values predicted by PB design model equation for all the medium components.

The maximum recombinant Cellulase A (CelA) production was obtained with the medium having the following composition (run No.3) per liter, namely Potassium di hydrogen phosphate -11g, Di ammonium hydrogen phosphate - 4g, Yeast extract-5g, Dextrose anhydrous -10g, Inoculum size – 5.0%, Temperature 36°C and Initial pH - 6.5. The minimum recombinant Cellulase A (Cel A) was obtained with the medium having the following composition (run No.7) per liter namely Potassium di hydrogen phosphate - 11g, Di ammonium hydrogen phosphate - 4g, Yeast extract-5g, Dextrose anhydrous -15g, Inoculum size – 2.5%, Temperature 38°C and Initial pH – 7.2. The variables namely concentration of Yeast Extract and percentage of inoculum size of fermentation process were found to be the most significant for recombinant Cellulase A (CelA) production as indicated by p-value <0.05 (Table-8,9&10). The statistical design of experiments offers efficient methodology to identify the significant variable and to optimize the factors with minimum number of experiments for recombinant Cellulase A (CelA) production by E.coli.

Potassium di hydrogen phosphate (g/L)	Di Ammonium hydrogen phosphate (g/L)	Yeast extract (g/L)	Dextrose anhydrous (g/L)	Inoculum size (%)	Temp (°C)	pH	OD	Cytosolic protein (g/L)
13	3	5	10	2.5	36	7.2	3.60	1.49
13	4	3	15	2.5	36	6.5	2.15	1.4
11	4	5	10	5	36	6.5	4.90	1.77
13	3	5	15	2.5	38	6.5	2.40	1.28
13	4	3	15	5	36	7.2	1.90	1.33
13	4	5	10	5	38	6.5	3.40	1.46
11	4	5	15	2.5	38	7.2	1.80	1.27
11	3	5	15	5	36	7.2	3.95	1.53
11	3	3	15	5	38	6.5	2.95	1.35
13	3	3	10	5	38	7.2	2.45	1.28
11	4	3	10	2.5	38	7.2	1.65	1.24
11	3	3	10	2.5	36	6.5	3.90	1.58

Table 6: Plackett-Burman design

1) Analysis of Variance:"

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Model	7	11.5765	99.56%	11.5765	1.65378	130.13	0.000
Linear	7	11.5765	99.56%	11.5765	1.65378	130.13	0.000
Potassium di hydrogen phosphate	1	0.8802	7.57%	0.8802	0.88021	69.26	0.001
Di Ammonium hydrogen phosphate	1	0.9919	8.53%	0.9919	0.99187	78.05	0.001
Yeast extract	1	2.1252	18.28%	2.1252	2.12521	167.23	0.000
Dextrose anhydrous	1	1.8802	16.17%	1.8802	1.88021	147.95	0.000
Inoculum size	1	1.3669	11.76%	1.3669	1.36688	107.56	0.000
Temperature	1	2.7552	23.70%	2.7552	2.75521	216.80	0.000
pH	1	1.5769	13.56%	1.5769	1.57687	124.08	0.000
Error	4	0.0508	0.44%	0.0508	0.01271		
Total	11	11.6273	100.00%				

Table 7: Factorial Regression: OD versus Potassium di, Di Ammonium, Yeast extract, Dextrose anhydrous, Inoculum size, Temperature, pH….

S	R-sq	R-sq (adj)	PRESS	R-sq (pred)
0.112731	99.56%	98.80%	0.4575	96.07%

2) Coded Coefficients:

Term	Effect	Coef	SE Coef	95% CI	T-Value	P-Value	VIF
Constant		2.9208	0.0325	(2.8305, 3.0112)	89.75	0.000	
Potassium dihydrogen phosphate	-0.5417	-0.2708	0.0325	(-0.3612, -0.1805)	-8.32	0.001	1.00
Di Ammonium hydrogen phosphate	-0.5750	-0.2875	0.0325	(-0.3779, -0.1971)	-8.83	0.001	1.00
Yeast extract	0.8417	0.4208	0.0325	(0.3305, 0.5112)	12.93	0.000	1.00
Dextrose anhydrous	-0.7917	-0.3958	0.0325	(-0.4862, -0.3055)	-12.16	0.000	1.00
Inoculum size	0.6750	0.3375	0.0325	(0.2471, 0.4279)	10.37	0.000	1.00
Temperature	-0.9583	-0.9583	0.0325	(-0.5695, -0.3888)	-14.72	0.000	1.00
pH	-0.7250	-0.3625	0.0325	(-0.4529, -0.2721)	-11.14	0.000	1.00

Table 8: Estimated effects and coefficients for OD600

Regression Equation in Uncoded Units

OD = 32.29 - 0.2708 Potassium di hydrogen phosphate - 0.5750 Di Ammonium hydrogen phosphate + 0.4208 Yeast extract - 0.1583 Dextrose anhydrous + 0.2700 Inoculum size - 0.4792 Temperature - 1.0357 pH

3) Analysis of Variance:

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Model	7	0.271800	99.23%	0.271800	0.038829	73.96	0.000
Linear	7	0.271800	99.23%	0.271800	0.038829	73.96	0.000
Potassium di hydrogen phosphate	1	0.020833	7.61%	0.020833	0.020833	39.68	0.003
Di Ammonium hydrogen phosphate	1	0.000133	0.05%	0.000133	0.000133	0.25	0.641
Yeast extract	1	0.032033	11.70%	0.032033	0.032033	61.02	0.001
Dextrose anhydrous	1	0.036300	13.25%	0.036300	0.036300	69.14	0.001
Inoculum size	1	0.017633	6.44%	0.017633	0.017633	33.59	0.004
Temperature	1	0.124033	45.28%	0.124033	0.124033	236.25	0.000
pH	1	0.040833	14.91%	0.040833	0.040833	77.78	0.001
Error	4	0.002100	0.77%	0.002100	0.000525		
Total	11	0.273900	100.00%				

Table 9: Factorial Regression: Cytosolic Protein versus Potassium di, Di Ammonium, Yeast extract, Dextrose anhydrous, Inoculum size, Temperature, pH….

S	R-sq	R-sq (adj)	PRESS	R-sq (pred)
0.0229129	99.23%	97.89%	0.0189	93.10%

4) Coded Coefficients:

Term	Effect	Coef	SE Coef	95% CI	T-Value	P-Value
Constant		1.41500	0.00661	(1.39664, 1.43336)	213.93	0.000
Potassium di hydrogen phosphate	-0.08333	-0.04167	0.00661	(-0.06003,-0.02330)	-6.30	0.003
Di Ammonium hydrogen phosphate	-0.00667	-0.00333	0.00661	(-0.02170, 0.01503)	-0.50	0.641
Yeast extract	0.10333	0.05167	0.00661	(0.03330, 0.07003)	7.81	0.001
Dextrose anhydrous	-0.11000	-0.05500	0.00661	(-0.07336,-0.03664)	-8.32	0.001
Inoculum size	0.07667	0.03833	0.00661	(0.01997, 0.05670)	5.80	0.004
Temperature	-0.20333	-0.10167	0.00661	(-0.12003,-0.08330)	-15.37	0.000
pH	-0.11667	-0.05833	0.00661	(-0.07670,-0.03997)	-8.82	0.001

Table 10: Estimated effects and coefficients for cytosolic protein

Regression Equation in Uncoded Units

Cytosolic protein (g/L) = 6.795 - 0.04167 Potassium di hydrogen phosphate -0.0067 Di Ammonium hydrogen phosphate + 0.05167 Yeast extract - 0.02200 Dextrose anhydrous + 0.03067 Inoculum size - 0.10167 Temperature - 0.1667 pH

5) Analysis of Variance:

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-V
Model	7	5.80023	99.46%	5.80023	0.82860	104.67	0.00
Linear	7	5.80023	99.46%	5.80023	0.82860	104.67	0.00
Potassium di hydrogen phosphate	1	0.19253	3.30%	0.19253	0.19253	24.32	0.00
Di Ammonium hydrogen phosphate	1	0.45630	7.82%	0.45630	0.45630	57.64	0.00
Yeast extract	1	0.85333	14.63%	0.85333	0.85333	107.79	0.00
Dextrose anhydrous	1	1.04430	17.91%	1.04430	1.04430	131.91	0.00
Inoculum size	1	1.30680	22.41%	1.30680	1.30680	165.07	0.00
Temperature	1	1.29363	22.18%	1.29363	1.29363	163.41	0.00
pH	1	0.65333	11.20%	0.65333	0.65333	82.53	0.00
Error	4	0.03167	0.54%	0.03167	0.00792		
Total	11	5.83190	100.00%				

Table 11: Factorial Regression: U/mL versus Potassium di, Di Ammonium, Yeast extract, Dextrose anhydrous, Inoculum size, Temperature, pH….

S	R-sq	R-sq (adj)	PRESS	R-sq (pred)
0.0889757	99.46%	98.51%	0.285	95.11%

Term	Effect	Coef	SE Coef	95% CI	T-Value	P-Value	VIF
Constant		4.8050	0.0257	(4.7337,4.8763)	187.07	0.000	
Potassium di hydrogen phosphate	-0.2533	-0.1267	0.0257	(-0.1980,-0.0554)	-4.93	0.008	1.00
Di Ammonium hydrogen phosphate	-0.3900	-0.1950	0.0257	(-0.2663,-0.1237)	-7.59	0.002	1.00
Yeast extract	0.5333	0.2667	0.0257	(0.1954,0.3380)	10.38	0.000	1.00
Dextrose anhydrous	-0.5900	-0.2950	0.0257	(-0.3663,-0.2237)	-11.49	0.000	1.00
Inoculum size	0.6600	0.3300	0.0257	(0.2587,0.4013)	12.85	0.000	1.00
Temperature	-0.6567	-0.3283	0.0257	(-0.3996,-0.2570)	-12.78	0.000	1.00
pH	-0.4667	-0.2333	0.0257	(-0.3046,-0.1620)	-9.08	0.001	1.00

Table 12: Estimated effects and coefficients for U/mL

Regression Equation in Uncoded Units

U/mL = 23.82 - 0.1267 Potassium di hydrogen phosphate - 0.3900 Di Ammonium hydrogen phosphate+ 0.2667 Yeast extract - 0.1180 Dextrose anhydrous + 0.2640 Inoculum size- 0.3283 Temperature - 0.6667 pH

6) Main Effects Plot for U/mL:

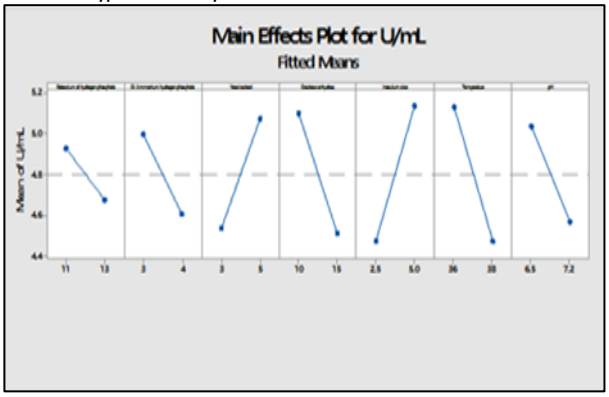

Fig. 2: Effects chart shows the significance of variables in PB experiments

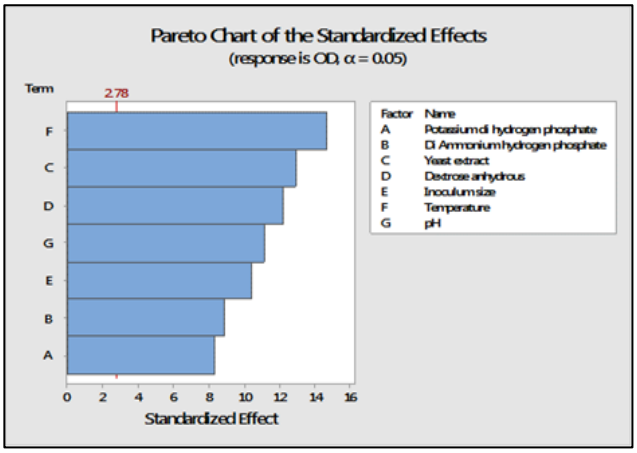

Fig. 3: Pareto chart of showed the significance of variables in PB experiments. Order of significance on OD

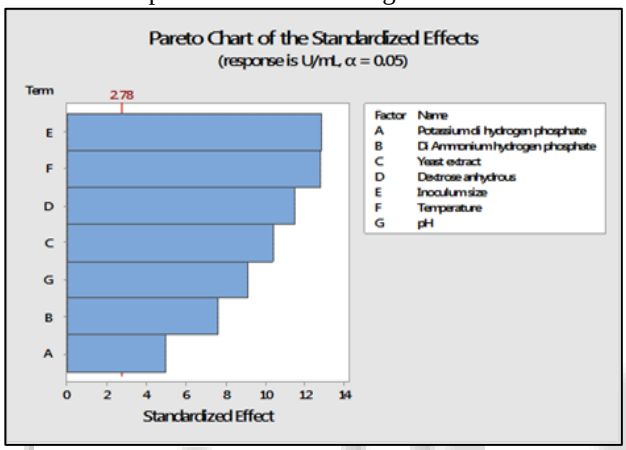

Fig. 4: Pareto chart of showed the significance of variables in PB experiments. Order of significance on U/mL

C. Optimization of significant variables for enhancement of Cel A Expression:

Using CCD different combinations of experiments were designed. The maximum and minimum values are denoted as (-1, 0 and +1) respectively (Table-13). The center point is the average of maximum and minimum values taken in the design Minitab-17 software. The effects of Inoculum size, Yeast Extract, IPTG Conc., Glucose and Induction temp on protein expression, as well as activity were experimentally studied and were 4.2.2.2 optimized using RSM. The range of values selected for the variables under study are as shown in Table-3. In order to study the interactions between these factors, 32 experiments were performed using different combinations (Table-14).

Run Order	Yeast Extract (g/L)	Inoculum size (%)	IPTG (mM)	Induction Temp (°C)	Dextrose (g/L)	Activity U/mL	Predicted U/mL
Variable Code	A	B	C	D	E		
1	3	2.5	0.25	24	8	2.78	2.751667
2	5	2.5	0.25	24	4	3.04	3.028333
3	3	5	0.25	24	4	4.05	4.055
4	5	5	0.25	24	8	7.3	7.298333
5	3	2.5	0.5	24	4	3.54	3.531667
6	5	2.5	0.5	24	8	4.19	4.175
7	3	5	0.5	24	8	4.76	4.761667
8	5	5	0.5	24	4	4.46	4.478333
9	3	2.5	0.25	26	4	3.4	3.395833
10	5	2.5	0.25	26	8	3.8	3.789167
11	3	5	0.25	26	8	4	4.005833
12	5	5	0.25	26	4	3.92	3.9425
13	3	2.5	0.5	26	8	4.8	4.7925
14	5	2.5	0.5	26	4	4.4	4.409167
15	3	5	0.5	26	4	4.3	4.325833
16	5	5	0.5	26	8	5.76	5.779167
17	2	3.75	0.375	25	6	3.55	3.5575
18	6	3.75	0.375	25	6	4.89	4.8775
19	4	1.25	0.375	25	6	3.77	3.810833
20	4	6.25	0.375	25	6	6.05	6.004167
21	4	3.75	0.125	25	6	3.29	3.304167
22	4	3.75	0.625	25	6	4.32	4.300833
23	4	3.75	0.375	23	6	3.9	3.9225
24	4	3.75	0.375	27	6	4.04	4.0125
25	4	3.75	0.375	25	2	4.79	4.764167
26	4	3.75	0.375	25	10	6.29	6.310833
27	4	3.75	0.375	25	6	5.33	5.3025
28	4	3.75	0.375	25	6	5.37	5.3025
29	4	3.75	0.375	25	6	5.34	5.3025
30	4	3.75	0.375	25	6	5.28	5.3025

Table 13: CCD design & results

1) Analysis of Variance:

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P V
Model	20	31.3804	99.92%	313804	1.56902	700.17	0.
Linear	5	14.9201	47.51%	14.9201	2.98402	1331.61	0.
Yeast Extract	1	2.6136	8.32%	2.6136	2.61360	1166.31	0.
Inoculum size	1	7.2161	22.98%	7.2161	7.21607	3220.15	0.
IPTG	1	1.4900	4.74%	1.4900	1.49002	664.92	0.
Ind. Temp	1	0.0121	0.04%	0.0122	0.01215	5.42	0.
Dextrose	1	3.5883	11.43%	3.5883	3.58827	1601.25	0.
Square	5	8.7606	27.90%	8.7606	1.75212	781.88	0.
YeastExtract*Yeast Extract	1	1.4697	4.68%	2.1582	2.15825	963.11	0.
Inoculum size*Inoculum size	1	0.0818	0.26%	0.2860	0.28605	127.65	0.
IPTG*IPTG	1	3.7204	11.85%	4.1250	4.12500	1840.77	0.
Ind. Temp*Ind. Temp	1	3.3875	10.79%	3.2674	3.26741	1458.07	0.
Dextrose*Dextrose	1	0.1012	0.32%	0.1012	0.10125	45.18	0.
2-Way Interaction	10	7.6996	24.52%	7.6996	0.76996	343.59	0.
YeastExtract*Inoculum size	1	0.7310	2.33%	0.7310	0.73103	326.22	0.
Yeast Extract*IPTG	1	0.3660	1.17%	0.3660	0.36603	163.34	0.
Yeast Extract*Ind Temp	1	0.3844	1.22%	0.3844	0.38440	171.54	0.
Yeast Extract*Dextrose	1	1.0920	3.48%	1.0920	1.09202	487.31	0.
Inoculum size*IPTG	1	0.9506	3.03%	0.9506	0.95062	424.21	0.
Inoculum size*Ind. Temp	1	1.8496	5.89%	1.8496	1.84960	825.38	0.
Inoculum size*Dextrose	1	0.9506	3.03%	0.9506	0.95063	424.21	0.
IPTG*Ind. Temp	1	1.1881	3.78%	1.1881	1.18810	530.19	0.
IPTG*Dextrose	1	0.0272	0.09%	0.0272	0.02723	12.15	0.
Ind.Temp*Dextrose	1	0.1600	0.51%	0.1600	0.16000	71.40	0.
Error	11	0.0246	0.08%	0.0246	0.00224		
Lack-of-Fit	6	0.0104	0.03%	0.0104	0.00173	0.60	0.
Pure Error	5	0.0143	0.05%	0.0143	0.00286		
Total	31	31.4050	100.00%				

Table 14: Response Surface Regression: U/mL versus Yeast Extract, Inoculum size, IPTG, Ind. Temp, Dextrose

S	R-sq	R-sq (adj)	PRESS	R-sq (pred)
0.0473382	99.92%	99.78%	0.297898	99.05%

Term	Effect	Coef	SE Coef	95% CI	T-Value	P-Valu
Constant		5.3025	0.0189	(5.2609, 5.3441)	280.83	0.000
Yeast Extract	0.66000	0.33000	0.00966	(0.30873, 0.35127)	34.15	0.000
Inoculum size	1.09667	0.54833	0.00966	(0.52707, 0.56960)	56.75	0.000
IPTG	0.49833	0.24917	0.00966	(0.22790, 0.27043)	25.79	0.000
Ind Temp	0.04500	0.02250	0.00966	(0.00123, 0.04377)	2.33	0.040
Dextrose	0.77333	0.38667	0.00966	(0.36540, 0.40793)	40.02	0.000
YeastExtract*Yeast Extract	-0.54250	-0.27125	0.00874	(-0.29049,-0.25201)	-31.03	0.000
Inoculum size*Inoculum siz	-0.19750	-0.09875	0.00874	(-0.11799,-0.07951)	-11.30	0.000
IPTG*IPTG	-0.75000	-0.37500	0.00874	(-0.39424,-0.35576)	-42.90	0.000
Ind Temp*Ind Temp	-0.66750	-0.33375	0.00874	(-0.35299,-0.31451)	-38.18	0.000
Dextrose*Dextros	0.11750	0.05875	0.00874	(0.03951, 0.07799)	6.72	0.000
YeastExtract*Inoculum size	0.4275	0.2138	0.0118	(0.1877, 0.2398)	18.06	0.000
YeastExtract*IPTG	-0.3025	-0.1513	0.0118	(-0.1773,-0.1252)	-12.78	0.000
Yeast Extract*Ind Temp	-0.3100	-0.1550	0.0118	(-0.1810,-0.1290)	-13.10	0.000
YeastExtract*Dextrose	0.5225	0.2612	0.0118	(0.2352, 0.2873)	22.08	0.000
Inoculum size*IPTG	-0.4875	-0.2438	0.0118	(-0.2698, -0.2177)	-20.60	0.000
Inoculum size*Ind. Temp	-0.6800	-0.3400	0.0118	(-0.3660, -0.3140)	-28.73	0.000
Inoculum size*Dextrose	0.4875	0.2437	0.0118	(0.2177, 0.2698)	20.60	0.000
IPTG*Ind. Temp	0.5450	0.2725	0.0118	(0.2465, 0.2985)	23.03	0.000
IPTG*Dextros	-0.0825	-0.0412	0.0118	(-0.0673,-0.0152)	-3.49	0.005
Ind.Temp*Dextrose	-0.2000	-0.1000	0.0118	(-0.1260,-0.0740)	-8.45	0.000

Table 15: Estimated Regression Coefficients for Activity U/mL

The statistical software Minitab 17 was used to generate a regression model for predicting the effect of combined parameters Inoculum size, Yeast Extract, IPTG, Dextrose anhydrous and Induction temp on the responses activity (U/mL) obtained from 32runs (table-13). To construct the response surface model, a second-order polynomial equation was fixed to the experimental data obtained using multiple regressions. The effect of combined parameters on activity of Cel A: The second-order polynomial equation for predicting the activity (U/mL) as a function of the five variables generated by CCD is.

U/mL (Y) = -241.35 + 5.404 Yeast Extract + 7.029 Inoculum size - 22.83 IPTG + 17.832 Ind Temp + 0.441 Dextrose - 0.27125 Yeast Extract*Yeast Extract - 0.06320 Inoculum size*Inoculum size - 24.000 IPTG*IPTG 0.33375 Ind Temp*Ind Temp+ 0.01469 Dextrose*Dextrose +0.17100 Yeast Extract*Inoculum size - 1.2100 Yeast Extract*IPTG - 0.1550 Yeast Extract*Ind Temp +0.13063 Yeast Extract*Dextrose - 1.5600 Inoculum size*IPTG - 0.27200 Inoculum size*Ind Temp + 0.09750 Inoculum size*Dextrose + 2.1800 IPTG*Ind Temp - 0.1650 IPTG*Dextrose - 0.05000 Ind Temp*Dextrose

Where U/mL (Y) is the predicted, from the variables of Inoculum size, Yeast Extract, IPTG, Dextrose anhydrous and Induction temp. For Equation, the values of the coefficients, t-values and P-values are listed in Table-15. The P-value level was 0.00, suggesting that the model was significant. The multiple correlation coefficient (R) was 0.992 for the activity and expression. The value of the adjusted R2 (0.9978) for Equation suggested that the model was capable of explaining 99% of the variation response.

R-Sq = 99.92% R-Sq (pred) = 99.05% R-Sq (adj) = 99.78%

The goodness of fit of the equation was determined by computing predicted activity yields. R2 value for the recombinant Cellulase (CelA) activity and expression was 99.92% and the significance of each parameter was analyzed using ANOVA table. For recombinant Cellulase (CelA) expression and activity with yeast extract, Inoculum size, IPTG, Induction Temperature and Dextrose anhydrous were observed to exert a significant effect and thus all the five variables have significant impact on recombinant Cellulase (CelA) production.

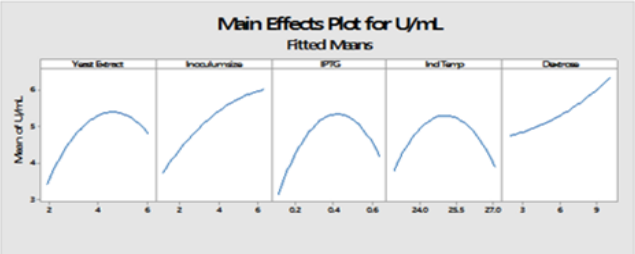

Fig. 5: Effects chart shows the significance of variables in CCD experiments

2) Response Surface Curves:

Three-dimensional response surface curves were obtained using from the Cel A production and activity values predicted by the CCD second order equations. Fig. 6 (a-j) shows the response surface curves for Cellulase A (Cel A) production and activity as a function of Yeast Extract, Dextrose anhydrous, Inoculum size, IPTG concentration and induction temperature of fermentation. When inoculum size of fermentation was increased from 2.5 to 5.0 %, the activity and production of cel A was increased as in Fig.-6 (c, e, f). Yield and activity of Cel A was slightly increased with increasing concentration of yeast extract from 2 to 5g/L and also with increase in Dextrose anhydrous concentration from 4 to 8g/l as in Fig.- 6 (b, d, e). but in case of increasing the IPTG concentration and increasing the Induction temperature was showing impact on the activity of Cel A.

In order to this results explains maintain the Ind.temparture and IPTG concentration slightly lower than the maximum values. (Fig . 6, g, h, I & j)

The optimum values obtained using RSM

Yeast Extract	=	5.0g/L
Inoculum size	=	5%
IPTG	=	0.25 mM
Induction Temperature	=	24°C
Dextrose anhydrous	=	8.0 g/L

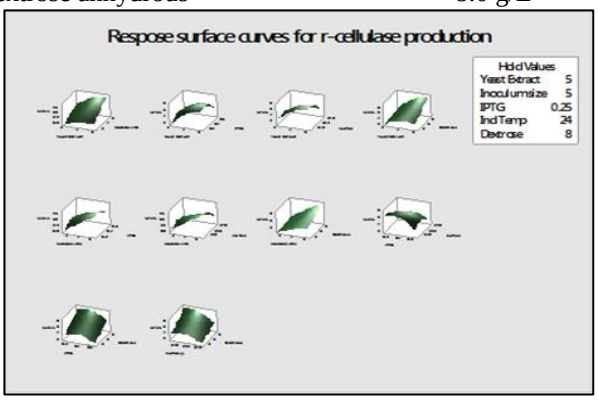

Fig. 6: Response surface curve for Cel A activity

D. Production of Cel A by Fed batch cultivation process:

To increase the volumetric cell yield a glucose-limited fed-batch cultivation mode was applied. Therefore, a 5.0 L stirred tank bioreactor equipped with optical sensors for pH and pO2 was filled with 1.0 L of cultivation medium. An initial glucose concentration of only 8 g L-1 secured that the oxygen transfer rate was sufficient to support fully aerobic growth during the initial batch phase. After 6.5 hours, when the initial glucose was exhausted, the pH and DO is getting increased start with continuous feeding of a highly concentrated glucose and yeast extract solution with a constant rate. The feeding rate was exponentially increased over the time to guarantee growth rate from initial 0.5ml/min to 1.25 ml /min. Consequently also the partial pressure of oxygen (pO2) decreased exponentially. When the pO2 reached the set point of 35%, a dual pO2 controller was started to maintain this level. Firstly, the air flow rate was increased to a maximum of 1.0 L min-1, and secondly the oxygen content was stepwise increased by pulses of pure oxygen to a maximum of 100% of the total gas flow rate. With this procedure, the culture grew to an optical density of OD600 = 58 at 36°C with a very small volume increase only (app. 150 ml, < 5%). An advantage of the stirred tank bioreactor system is the reliable temperature control, also at higher cell densities when the metabolic rates are high. after attaining OD600=58 , the fermentor temperature was reduced to 24°C and the culture was induced with 0.25mM IPTG , continued up to 4hrs. The maximum OD600 was obtained 69. It is observed that 70 - 75 g of solid biomass is obtained from 1 L of fermentor broth, approx.2.8 g/L r-CelA protein achieved (estimated by densitometry) and finally 7.9.U/mL activity was observed by optimized fermentation process (CelA assay).

Batch No.	Cell Biomass (g/L)	Cytosolic protein (g/L)	Activity (U/mL)
1	72	1.88	7.7
2	75	1.9	7.9
3	69	1.81	7.5

Table 16: Optimization of Fermentation process for improve active Cel a production

E. Homogenization of cell biomass:

1) Preparation of cell suspension:

E.coli biomass fresh / frozen-thawed was weighed and transferred in to a reactor/mixing vessel and the buffer is added to make up 10% cell suspension (w/v). Then mixture kept for uniform mixing subjected to homogenization for getting lysate solution. The obtained lysate has been again homogenized for 5- 6 cycles with addition of lysis buffer to make final volume 5-10% suspension. The cell lysate to be kept at 10-15°C under uniform mixing till next step.

2) Cell Lysis by Homogenization:

Dissolve the Tris base in DM /Purified-water/WFI in the final volume to 90% (900 ml). Adjust the pH to 8.0 and then make the final volume to 100%.

Operation: Nero-High pressure homogenizer was used for homogenization with below parameters.

Operating pressure: 700 - 750 bar
No. of Cycles: 3-4
Final OD_{600}: Below 5.0
Temperature: 10-15°C
Procedure:

Maintain the temperature of cell suspension at 10-15°C in the holding reactor using chilled water/brine solution which further facilitate in maintaining overall temperature. The cell suspension sample passed through homogenizer to lyse the cells at 700-750 bar for 3-4 No. of cycles. Then the lysate sample should be less than 5.0 OD.

F. Clarification by TFF (Tangential Flow Filtration):

The cell lysate obtained through homogenization was passed through 0.45µ TFF Cassettes system and permeate was collected by maintaining trans-membrane pressure (TMP) between 0.5-0.7 bar. To maintain TMP, feed and retentate line pressure was maintained between 1-1.5 bar. Permeate to retentate ratio achieved was 3:1. Further, diafiltration was performed with retentate by adding 1:1 volume of the lysis buffer for 4 cycles of diafiltration to extract out > 75% of protein. During the entire operation, the temperature should be maintained at 10-15°C by controlling the jacket temperature. Typically 1.0 L of lysate can be made to ~ 0.4 L retentate and ~1.4 -1.6L pooled permeates by doing diafiltration with the buffer. Pooled Permeate was loaded on to affinity column.

G. Affinity chromatography based purification of Cellulase (CelA)A using Ni-NTA resins:

A common technique involves engineering a sequence of 6 to 8 histidine into the N- or C-terminal of the protein. The polyhistidine binds strongly to divalent metal ions such as nickel. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column. The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, or by a decrease in pH (typically to 4.5), which decreases the affinity of the tag for the resin. While this procedure is generally used for the purification of recombinant proteins with an engineered affinity tag (such as a 6xHis tag or Clontech's HAT tag), it can also be used for natural proteins with an inherent affinity for divalent cations.

All the eluted samples from affinity column chromatography were subjected to SDS-PAGE analysis for the determination of fractions with over expressed recombinant Cellulase A (Cel A)

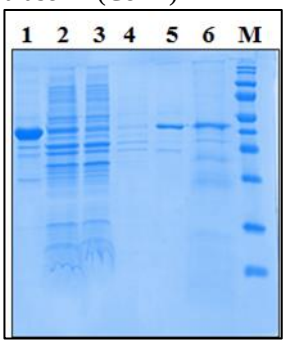

Fig. 7: Affinity chromatography based purification of Cellulase A (CelA) using Ni-NTA resins: Lane Samples: M: Protein marker, L: Load (E.coliBL21DE3-pRSETA-CelA-Induced Lysate) ,FT : Flow through, W1 :Wash1 (10mM Imidazole), W2:Wash2 (10mM Imidazole), E1:Elution1 (250mM Imidazole), E2:Elution2 (250mM Imidazole)

H. Zymogram analysis:

In a screen for cellulolytic activity in column eluted samples, we detected high levels of activity against CMC in 200mM imidazole column elution obtained, from IPTG induced E.coli expressed Cellulase A (CelA). We therefore were interested in testing differential levels of expression of enzymes responsible for cellulose breakdown. In order to ascertain whether similar cellulolytic systems were involved in activity During development, we used zymogram with Ni-NTA column eluted samples from E.coli based expressed to detect proteins having activity against CMC substrate (Fig. 8). In these assays, we detected a similar pattern of Cellulase (CelA) activities in samples from different developmental stages. The Cellulase A (CelA) activities, corresponding to proteins of ~ 50 kDa in molecular mass, were observed for all samples.

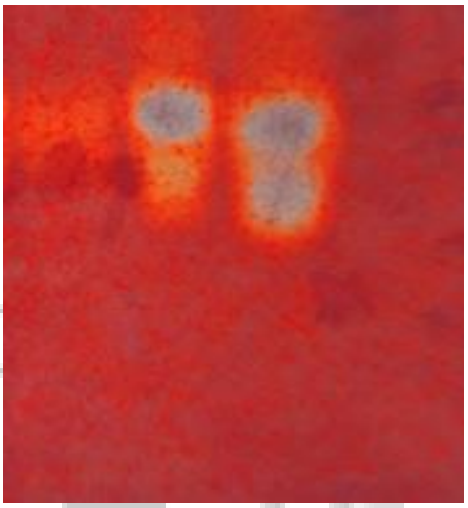

Fig. 8: Lane No.: 1.Negative control; 2. Positive control; 3.Purified Cel A; M; protein Maker

A test for CMCase activity was performed on the proteins separated by SDS-PAGE. The major CMCase activity was associated with ~50KDa protein.

I. Cellulase (CelA) A assay:

1) Quantification of recombinant Cellulase A (CelA) Produced by E.coli:

r- Cellulase (CelA) (endoglucanase) produced by E.coli was quantified by performing reducing sugar assay and filter paper and CMC (Carboxy methyl Cellulase A (CelA) as substrate. Maximum endoglucanase enzyme activity of 7.9 U/ml was obtained. Quantification results confirmed the potential production of recombinant Cellulase A (CelA) by E.coli.

2) Characterization of recombinant Cellulase (CelA)

a) Effect of Temperature on the Activity of Purified Cellulase (CelA)s:

Column purified recombinant Cellulase (CelA) was incubated at various temperatures ranging from 10°C to 100°C to observe changes in temperature range after purification. The optimum temperature for endoglucanase from 45°C (crude optimum) to 50°C. Moreover the purified enzymes were active over a wider temperature range from 30°C to 65°C. Endoglucanase maintained 82.5% of its activity at 30°C and 82.3% at 60°C, and dropped to 29.1% on further increase in temperature.

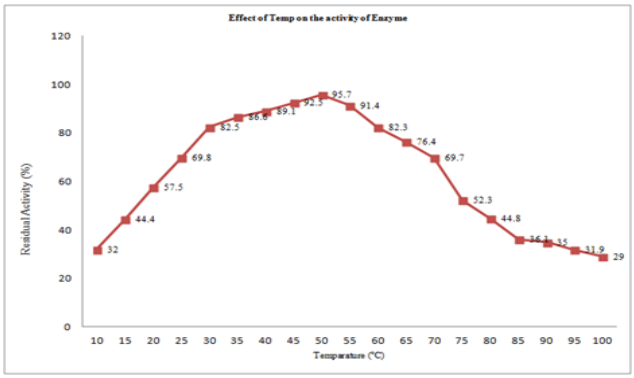

Fig. 9: Effect of Temperature on the Activity of Cellulase (CelA) enzyme

b) Effect of pH on the Activity of Crude Cellulase (CelA)s:

The activities of endoglucanase was assayed at different pH ranging from 2-9.Data indicated that purified enzyme was active at pH ranging from 5.0 to 6.0.Decrease in activity was noted at both alkaline and acidic ranges. Maximum activity of the enzyme was at pH 5.5. Endoglucanase retained 62.0% of its residual activity at pH4.0 in the acidic range and at pH 9.0 still had 31.0% of its residual activity. Endoglucanase was slightly tolerant towards acidic and more at alkaline range and retained 29% of its residual activity at pH2.5and 31.8% at pH9.0.

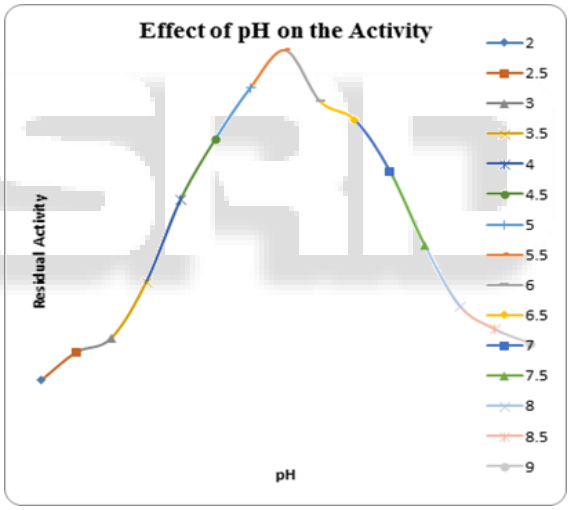

Fig. 10: Effect of pH on the Activity of Cellulase (CelA) enzyme

c) Effect of Metal Ions on the Activity of purified Cellulase (CelA):

Effect of various metal ions at 1mM final concentrations on the activity of Endoglucanase, was observed following incubation of enzyme mixture with respective metal ions at 50 °C for 30 minutes. Calculated residual activities revealed that Mn2+, Zn2+, Ca2+, Mg2+, K, and Na ions had stimulatory effect on the activity of recombinant Cellulase (CelA) with maximum residual activities for endoglucanase at 149.9%, 144.16%, 130.2%, 127.9%, 122.5%, and 104.9% respectively. Fe2+ ion neither inhibited nor had a stimulatory effect on endoglucanase with residual activities of 97.53%. Maximum inhibitory effect on activity of endoglucanase was exhibited by Cu2+with residual activity of 46.6% followed by Pb53.6% and Hg 62.4%. Least inhibition was reported for endoglucanase by Co 2+at 85.5% residual activity.

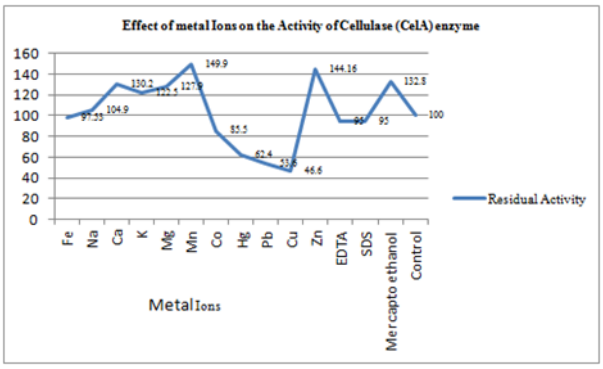

Fig. 11: Effect of metal Ions on the Activity of Cellulase (CelA) enzyme

Mercaptoethanol a thiol compound that inhibits oxidation of sulfhydryl residue stimulated activity of endoglucanses132.8%. EDTA a metal chelator can remove metals that effect enzyme activity and SDS a strong surfactant that can cause protein to denature had a slight inhibitory effect. The enzyme retained more than 95% of their residual activity in EDTA and SDS.

IV. Conclusion

The use of lingocellulosic materials for second generation ethanol production would give several advantages such as minimizing the conflict between land use for food and fuel production, providing less expensive raw materials than conventional agricultural feedstock, allowing lower greenhouse gas emissions than those of first generation ethanol. However, cellulosic biofuels are not produced at a competitive level yet, mainly because of the high production costs of the cellulolytic enzymes. This study suggested that the recombinant Cel A can be produced with reasonably good yields and as a soluble and active form in E.coli expression host at fermentation level, which can be potentially scaled up to industry. This system will offer a platform for conversion of abundant renewable biomass to biofuels and biorefinery products.

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