Process Evaluation of Recombinant Chitin Deacetylase Expression in *E. Coli* Rosetta pLysS Cells using Statistical Design of Experiments

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Abstract

Chitin is a natural polymer with *N*-acetylglucosamine units, extracted from seafood waste as a major source. It remains an underexplored polymer due to its crystalline structure. The commercial applicability can be improved if we could make it soluble. One of the routes employed to decrease this crystallinity is the conversion of chitin to chitosan via deacetylation. The industrial production of chitosan uses chemical methods, which leaves the process footprint on the environment. The greener alternative approach to producing chitosan is using chitin deacetylases (CDA). The enzymatically converted chitosan with known characteristics has a wide range of applications, importantly in the biomedical field. In the present paper, we report heterologous expression of CDA from a marine moneran; *Bacillus aryabhattai* B8W22. The process and the nutritional conditions were optimized for the submerged fermentation condition of *E. coli* Rosetta pLysS expressing the recombinant CDA using the design of experiment tools. The employment of central composite design (CCD) resulted in a ~2.39 fold increase in the total activity of expressed CDA with the process conditions of induction temperature at 22 ºC, agitation at 120 rpm, and 30 h of fermentation. The nutritional conditions required for the optimized expression were 0.061% glucose concentration and 1% lactose in media. The employment of these optimal growth conditions could result in cost-effective large-scale production of the lesser-explored moneran deacetylase, embarking on the greener route to produce biomedical grade chitosan.

Introduction

Chitin, a structural β 1-4 glycosidic linked natural polymer, is the second most abundant after cellulose. The presence of chitin is apparent as a structural support component in the cuticle of insects, shells of crustaceans, and the cell wall of fungi (Sudha et al. 2014). The large-scale commercial extraction of chitin is performed using shells of crab and shrimps. Chitin is insoluble in most of the solvents, which limits its industrial application (Yadav et al. 2019). The *N*-deacetylation of chitin results in imparting a positive charge and increases its solubility. This deacetylated polymer of chitin i.e., chitosan being biodegradable, biocompatible, non-toxic, environment-friendly, bio-functional, and bio-renewable has wide applications in food, textile, agriculture, and biomedical field (Yadav et al. 2019). Chitin can be modified to chitosan using two different routes: chemical and enzymatic. Biomedical application of chitosan requires controlled and defined deacetylation which is restricted in chemical conversion. This limitation can be addressed by the use of chitin deacetylases (CDAs), a green enzymatic route for the deacetylation process. (Cord-Landwehr et al. 2020; Wattjes et al. 2020).

Naturally, CDA is found in all the 5 kingdoms of life (Chavan and Deshpande 2013). Chitin deacetylase belongs to carbohydrate esterase family 4 (CE-4) defined in the CAZY database. The enzymes of the CE-4 family share the NodB homology domain or polysaccharide deacetylase domain as a conserved region. CDA hydrolysis acetamido group in *N*-acetylglucosamine units of chitin converting it to chitosan containing glucosamine unit with acetic acid as a by-product (Urch et al. 2009).

Even though CDAs are present naturally, the extraction and purification of the wild-type enzymes are not economical due to the involvement of multiple steps and their low yield. The fragility of the enzyme diminishes its activity further at the end of this entire extraction process. Recombinant DNA technology is the gold standard for the industrial production of an enzyme. This technique is cost-effective, easy to handle, and feasible for large-scale production. Research groups have worked on the heterologous expression of the CDA gene in *Escherichia coli* optimizing the production in media components creating media ranging from Luria-Bertani (LB) to terrific broth (TB) (Grifoll-Romero et al. 2018). The inclusion of glycerol in the terrific broth has been shown to improve the yields of the recombinant proteins (Blommel et al. 2007). In most of the *E. coli* expression studies, Isopropyl ß-D-1-thiogalactopyranoside (IPTG) is used as an inducer that requires regular monitoring of absorbance at 600nm of the fermenting broth. This regular intervention during the fermentative process also opens up avenues for contamination. An alternative to address these limitations is the inclusion of lactose in the media instead of IPTG. The *E. coli* consumes this lactose and produces allo-lactose as a by-product, resulting in an auto-induction condition. The process can be made more cost-effective with the inclusion of process optimization. Central composite design (CCD) of response surface methodology (RSM) is a statistically proven optimization method. As several factors affect the recombinant enzyme expression, CCD helps to optimize the expression condition statistically. In the present study, we have isolated halophilic bacteria from the sea sediments of Arabian Sea expressing chitin deacetylase. The CDA gene was identified, amplified, and cloned into the pET-22b vector. The heterologous CDA expression in *E. coli* Rosetta pLysS cells was optimized by CCD. The process parameters chosen were induction temperature, agitation rate, incubation time, glucose and lactose concentration. Further, the CDA expression was evaluated with the predicted optimized condition and cross validated using SDS PAGE. Finally, the point of lactose induction was determined by studying the fermentation kinetics of glucose concentration, biomass, and total enzyme activity during the expression.

Materials And Methods

Isolation, screening, and identification

A 40 m sea sediment sample was collected from the Arabian sea. The corresponding location was identified with coordinates 12°48' N and 74°40'E. Microorganisms were isolated from the sample by serial dilution method under the halophilic condition on a colloidal chitin plate containing synthetic seawater. The microorganisms were screened for chitin deacetylase activity on a receptor-based screening plate (Pawaskar et al. 2019). The positively screened microorganisms were quantified for their chitin deacetylase activity and identified by molecular identification.

Gene identification, and cloning

An isolate having maximum chitin deacetylase activity was identified and its CDA gene was predicted by homology-based comparison. A reference putative polysaccharide deacetylase gene was retrieved from the NCBI gene database. The reference gene was searched in identified isolate's whole genome by genome BLAST server. Thus, the identified gene was annotated as the CDA gene in the NCBI gene data bank. A set of primers were designed to amplify the gene by inserting *Nde*I coding site at the 5' end and *Xho*I coding site at the 3' end. To clone, the amplified gene into pET-22b plasmid, they were digested with

*Nde*I and *Xho*I, ligated using a DNA ligase enzyme. The successful cloning was confirmed by colony PCR with gene-specific primers, restriction digestion, and sequencing with T7 universal primer.

Extracellular expression of Ba**CDA**

The pET-22b *Ba*CDA vector was transformed into *E. coli* Rosetta pLysS cells by calcium chloride method to express *Ba*CDA gene. The initial expression was carried out in TB media (composition % (w/v): 1.2, tryptone; 2.4, yeast extract; 1X TB salt, and 0.6% glycerol) containing 0.05% (w/v) glucose and 0.2% (w/v) lactose at 16°C with 180 rpm agitation rate till its growth reaches to stationary phase (Studier 2005). The expression was auto-induced by including lactose and glucose in the media as inducer and repressor respectively. To enhance the expression and enzyme activity using the statistical approach (CCD), three physical factors (induction temperature, agitation rate, and induction time) and two nutritional factors (glucose and lactose concentration) were chosen.

Optimization and experimental design

In this study, CCD was framed using a statistical optimization tool MINITAB 17.0 (Trial Version) to design the experiments and fit the second-order polynomial model. Five factors at five different levels (-2, -1, 0, +1, +2) with six replicates at the center point requiring 32 experimental runs were designed. The five factors such as induction temperature, agitation rate, incubation time, glucose concentration, and lactose concentration were selected for optimization of expression of recombinant chitin deacetylase. These factors were selected based on our preliminary studies. Table 1 depicts the factors and their levels both in coded and uncoded terms used in the process optimization using CCD. Whereas Table 2 depicts the experimental design matrix, levels, and factors in terms of coded and uncoded units. Further to understand the impact of factor interactions, a quadratic model was established to correlate the total activity and expression of recombinant chitin deacetylase in *E. coli* Rosetta pLysS cells and is shown below:

Table 1
The factors and their levels in coded and uncoded terms used in experimental design to estimate the expression of recombinant chitin deacetylase in *E. coli* Rosetta pLysS cells.

Factors	Symbol	Coded and Uncoded values				
		-2	-1	0	+1	+2
Induction temperature, °C	A	16	20	24	28	32
Agitation rate, rpm	B	80	120	160	200	240
Induction time, h	C	12	20	28	36	44
Glucose concentration, % (w/v)	D	0	0.025	0.05	0.075	0.1
Lactose concentration, % (w/v)	E	0.2	0.4	0.6	0.8	1

Table 2
Experimental design matrix with experimental and predicted total activity (expression) of recombinant chitin deacetylase in *E. coli* Rosetta pLysS cells.

Run order	A	B	C	D	E	Total activity (U/L)	
						Experimental	Predicted
1	20 (-1)	120 (-1)	20 (-1)	0.025 (-1)	0.8 (+1)	49.74 ± 1.87	52.50
2	28 (+1)	120 (-1)	20 (-1)	0.025 (-1)	0.4 (-1)	47.44 ± 1.72	35.46
3	20 (-1)	200 (+1)	20 (-1)	0.025 (-1)	0.4 (-1)	27.71 ± 1.35	-5.06
4	28 (+1)	200 (+1)	20 (-1)	0.025 (-1)	0.8 (+1)	39.96 ± 1.48	46.65
5	20 (-1)	120 (-1)	36 (+1)	0.025 (-1)	0.4 (-1)	60.12 ± 1.56	50.01
6	28 (+1)	120 (-1)	36 (+1)	0.025 (-1)	0.8 (+1)	9.56 ± 0.78	19.10
7	20 (-1)	200 (+1)	36 (+1)	0.025 (-1)	0.8 (+1)	43.48 ± 1.74	58.24
8	28 (+1)	200 (+1)	36 (+1)	0.025 (-1)	0.4 (-1)	10.70 ± 1.35	30.54
9	20 (-1)	120 (-1)	20 (-1)	0.075 (+1)	0.4 (-1)	23.50 ± 1.75	23.92
10	28 (+1)	120 (-1)	20 (-1)	0.075 (+1)	0.8 (+1)	57.66 ± 0.17	70.97
11	20 (-1)	200 (+1)	20 (-1)	0.075 (+1)	0.8 (+1)	25.04 ± 2.02	32.15
12	28 (+1)	200 (+1)	20 (-1)	0.075 (+1)	0.4 (-1)	99.33 ± 2.16	82.42
13	20 (-1)	120 (-1)	36 (+1)	0.075 (+1)	0.8 (+1)	127.86 ± 1.07	118.55
14	28 (+1)	120 (-1)	36 (+1)	0.075 (+1)	0.4 (-1)	37.05 ± 0.97	23.55
15	20 (-1)	200 (+1)	36 (+1)	0.075 (+1)	0.4 (-1)	54.67 ± 1.16	60.98
16	28 (+1)	200 (+1)	36 (+1)	0.075 (+1)	0.8 (+1)	35.35 ± 0.68	34.74
17	16 (-2)	160 (0)	28 (0)	0.050 (0)	0.6 (0)	1.27 ± 0.36	9.87
18	32 (+2)	160 (0)	28 (0)	0.050 (0)	0.6 (0)	2.92 ± 0.04	-2.08
19	24 (0)	80 (-2)	28 (0)	0.050 (0)	0.6 (0)	61.65 ± 1.17	69.27
20	24 (0)	240 (+2)	28 (0)	0.050 (0)	0.6 (0)	59.95 ± 1.97	55.92
21	24 (0)	160 (0)	12 (-2)	0.050 (0)	0.6 (0)	3.54 ± 0.77	17.40
22	24 (0)	160 (0)	44 (+2)	0.050 (0)	0.6 (0)	41.86 ± 1.07	31.58
23	24 (0)	160 (0)	28 (0)	0 (-2)	0.6 (0)	36.31 ± 1.15	35.13
24	24 (0)	160 (0)	28 (0)	0.100 (+2)	0.6 (0)	70.33 ± 2.34	75.10
25	24 (0)	160 (0)	28 (0)	0.050 (0)	0.2 (-2)	115.46 ± 2.27	143.00
26	24 (0)	160 (0)	28 (0)	0.050 (0)	1 (+2)	199.72 ± 1.14	175.77
27	24 (0)	160 (0)	28 (0)	0.050 (0)	0.6 (0)	113.24 ± 0.88	121.80
28	24 (0)	160 (0)	28 (0)	0.050 (0)	0.6 (0)	123.24 ± 1.88	121.80
29	24 (0)	160 (0)	28 (0)	0.050 (0)	0.6 (0)	133.24 ± 1.62	121.80
30	24 (0)	160 (0)	28 (0)	0.050 (0)	0.6 (0)	124.24 ± 2.12	121.80
31	24 (0)	160 (0)	28 (0)	0.050 (0)	0.6 (0)	122.24 ± 0.88	121.80
32	24 (0)	160 (0)	28 (0)	0.050 (0)	0.6 (0)	118.24 ± 3.12	121.80

Where A: Induction temperature, °C; B: Agitation rate, rpm; C: Induction time, h; D: Glucose concentration, %(w/v); E: Lactose concentration, % (w/v). All the runs were performed in triplicates and the value was represented in average with standard deviation.

$$Z = \phi_0 + \sum_{k=1}^{5} \phi_k y_k + \sum_{k=1}^{5} \phi_{kk} y_k^2 + \sum_{k=1}^{4} \sum_{l=k+1}^{5} \phi_{kl} y_k y_l$$

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Where: Z, the total activity of recombinant chitin deacetylase (predicted response); y_k and y_l are the independent factors, ϕ_0, intercept term; ϕ_k, linear effect; ϕ_{kk}, squared effect, and $\phi_k \phi_l$, interaction effects. The regression equation 1 was designed and analyzed by using the software MINITAB 17.0.

Validation of model

The optimized process parameters obtained for the expression of CDA in *E. coli* Rosetta pLysS cells were validated by the Minitab response optimizer tool available in MINITAB 17.0 (Trial Version). The experiments were carried out in triplicated and the experimental total activity was compared with predicted total activity under optimized conditions.

Analytical Methods

Expression, biomass, and protein quantification

The expression level was analyzed by using ImageJ software followed by running 12% SDS-PAGE. The biomass was calculated at the end of each experimental run by weighing the cell pellet. To each gram of cell pellet 5 mL of lysis buffer was added (50mM Tris-HCl, 300mM NaCl, 10mM Imidazole) and the soluble protein was collected by disrupting cell pellet by sonication, for 10 cycles with pulse 10s on and 10s off at 60% amp. The lysate was collected by centrifuging at 8000 rpm for 10 min at 4°C. The protein was quantified by Bradford's assay method. The *Ba*CDA activity was also determined in the lysate using an acetate assay kit.

Enzyme activity assay

The chitin deacetylase activity was determined using an acetate assay kit as per the user manual. Briefly, ethylene glycol chitin (1mg/mL) (EGC) was used as a substrate (Schomburg and Salzmann 1991). In 100 µL of reaction, 40 µL of the substrate with 20 µL of lysate in presence of 40 µL of 50 mM Tris-HCl (pH: 7) buffer was incubated for one hour at 30°C with mixing at 800 rpm. After the incubation time, the reaction was stopped by separating the enzyme using a 3 kDa spin column. 10 µL of the reaction mixture was used for assay and enzyme activity was calculated accordingly. One Unit of the enzyme is defined as the activity which released 1µmol of acetate from the substrate per microliter of enzyme per minute. The enzyme activity assay was carried out in triplicates and the respective enzyme activity was calculated accordingly.

Fermentation kinetics of lactose induction

To investigate the point of lactose induction and expression start point, activity profiling was done. At the optimized condition, the expression was repeated. During the expression, glucose concentration using glucose estimation kit, biomass by weighing cell pellet, and enzyme activity using acetate assay kit were determined. The kinetics of glucose concentration, biomass yield, and enzyme activity were evaluated.

Results

Isolation, screening, and identification

The isolation of the microbes from the Arabian Sea sediment yielded fifteen bacterial and one fungal colony. The bacterial colonies were subjected to four rounds of purification on the colloidal chitin agar plate to obtain a single isolated colony. The isolated colonies were spot inoculated on a receptor-based screening plate (Pawaskar et al. 2019). Four positive isolates were picked up based on the fluorescence emission signifying the CDA activity. The activity was later quantitated spectrophotometrically. Based on these results, the isolate MS7 was selected for molecular characterization. The 16S rRNA sequence analysis of isolate MS7 indicated towards *Bacillus aryabhattai* B8W22 strain with a similarity of 99.84%. The phylogenetic tree was constructed by selecting homologous sequences with *Bacillus niacin* as the outgroup. *Bacillus aryabhattai* B8W22 was deposited at National Culture for Microbial Resource (NCMR), India with the accession ID: MCC 3987 (Pawaskar et al. 2021).

Gene identification, and cloning

A putative polysaccharide deacetylase gene from *Bacillus megaterium* with Gene ID: NZ_CP009920.1 was taken as a reference gene. The gene was probed in the *Bacillus aryabhattai* whole genome with Genome ID: NZ_JYOO01000001.1 using genome BLAST server. The result yielded a 100% query coverage with a 97% match. The sequence was annotated in the NCBI gene databank in the third-party section of the DDBJ/ENA/GenBank databases with accession number TPA: BK010747 (Pawaskar et al. 2021).

Using the designed primers, *Ba*CDA (~765bp) was amplified at optimized PCR conditions. The genetic code of *Ba*CDA was affirmed by Sanger sequencing employing the T7promoter and the terminator region of the vector. Post confirmation, the vector construct was transformed into *E. coli* Rosetta pLysS cells for expression.

Extracellular expression of Ba**CDA**

In TB basal media, the concentration of glucose depleted completely within 24 h of fermentation. This is followed by a second lag phase of 4 h, and further lactose consumption and auto-induction was initiated in the second log phase which prolonged for an additional 20 h. The maximum biomass yield and total CDA activity were found to be 22.26 ± 0.98 g/L and 84.67 ± 0.56 U/L respectively in the stationary phase at 52 h of fermentation (Pawaskar et al. 2021).

Process optimization of expression of extracellular recombinant enzyme chitin deacetylase in E. coli **using central composite design (CCD)**

In the current study, five factors induction temperature (A), agitation rate (B), induction time (C), glucose concentration (D), and lactose concentration (E) were considered (Table 1). Accordingly, A, B, C, D, and E were taken as exogenous factors while the total activity of expression of extracellular recombinant chitin deacetylase was chosen as the endogenous factor (response). The expression of extracellular recombinant enzyme chitin deacetylase in *E. coli* Rosetta pLysS cells is quantified and represented as "total activity (U/L)" of recombinant chitin deacetylase (Table 2). The results of biomass yield, expression of CDA, total protein content, specific activity at all the experimental runs are shown in Table 3. The expression was estimated by SDS-PAGE and quantified using ImageJ software. The total activity at each condition was determined using acetate assay and represented along with the SDS-PAGE as shown in Fig. 1.

Table 3
The results of biomass yield, expression of CDA, total protein content, the specific activity of all the experimental runs given by central composite design.

Run order	Biomass (g/L)	Expression	Total protein (mg/L)	Specific activity (U/mg)
1	11.60 ± 0.30	1.000	2280.20 ± 103.90	0.022
2	10.20 ± 0.60	0.992	2083.50 ± 99.75	0.023
3	16.54 ± 1.27	0.887	2697.80 ± 107.40	0.010
4	14.80 ± 0.90	0.923	2639.70 ± 65.35	0.015
5	11.88 ± 0.94	1.115	2288.40 ± 35.20	0.026
6	13.36 ± 1.18	0.739	2618.00 ± 98.10	0.004
7	20.98 ± 0.99	0.973	4766.00 ± 84.70	0.009
8	17.88 ± 0.06	0.810	3430.80 ± 28.90	0.003
9	9.30 ± 0.35	0.830	1794.00 ± 92.26	0.013
10	8.60 ± 0.70	1.051	1706.60 ± 22.00	0.034
11	17.20 ± 0.90	0.848	2916.00 ± 77.16	0.009
12	12.60 ± 0.20	1.229	2353.00 ± 50.18	0.042
13	14.80 ± 0.60	1.420	2652.00 ± 99.88	0.048
14	12.72 ± 1.36	0.919	2369.40 ± 49.80	0.016
15	18.04 ± 0.48	1.042	3741.70 ± 99.65	0.015
16	17.94 ± 1.03	0.894	3528.30 ± 74.93	0.010
17	6.60 ± 0.70	0.517	1663.00 ± 55.44	0.001
18	16.62 ± 0.69	0.653	2842.20 ± 58.60	0.001
19	9.66 ± 0.67	1.160	1816.10 ± 101.65	0.034
20	18.46 ± 0.77	1.088	3810.60 ± 73.75	0.016
21	11.02 ± 0.99	0.718	2176.80 ± 50.40	0.002
22	12.76 ± 0.62	0.965	2391.60 ± 36.16	0.018
23	12.94 ± 1.03	0.913	2460.90 ± 118.65	0.015
24	12.20 ± 0.90	1.184	2308.50 ± 100.25	0.030
25	10.98 ± 0.51	1.333	2167.20 ± 99.90	0.053
26	13.04 ± 0.48	1.522	2544.10 ± 44.85	0.079
27	12.58 ± 0.71	1.313	2338.80 ± 97.50	0.048
28	12.30 ± 0.85	1.131	2358.80 ± 111.50	0.052
29	12.80 ± 0.60	1.282	2321.80 ± 55.19	0.057
30	12.45 ± 0.78	1.818	2345.80 ± 94.39	0.053
31	12.65 ± 0.68	1.133	2319.80 ± 56.09	0.053
32	12.72 ± 0.64	1.003	2342.80 ± 45.74	0.050
All the runs were performed in triplicates and the value was represented in average with standard deviation.				

Initially, the full second order regression model obtained for total activity of recombinant chitin deacetylase was significant with high coefficient of determination (R^2) of 0.9595. Whereas the predicted R^2 was 0%, indicating the lack of predictability of a model and their regression equation is given below:

Z_1 (Total activity, U/L) = -1844 + 99.7 A + 2.64 B + 36.33 C+ 808 D + 55 E - 1.842 A^2 - 0.00925 B^2 - 0.3801 C^2 - 26676 D^2 + 234.9 E^2 + 0.0562 A x B - 0.609 A x C + 44.8 A x D - 10.32 A x E - 0.0203 B x C + 0.83 B x D - 0.979 B x E + 28.2 C x D + 3.10 C x E + 432 D x E. (2)

Where A: Induction temperature, °C; B: Agitation rate, rpm; C: Induction time, h; D: Glucose concentration, %(w/v); and E: Lactose concentration, % (w/v).

The results of the analysis of variance (ANOVA) of the full model (Equation 2) are given in Table 4.

Table 4
Analysis of variance (ANOVA) for the full model.

Source	DF	Seq SS	Contribution, %	Adj SS	Adj MS	F-value	P-value	
Model	20	70183.3	95.95	70183.3	3509.2	13.03	0	S
Linear	5	4790.8	6.55	4790.8	958.2	3.56	0.037	S
A	1	214.6	0.29	214.6	214.6	0.8	0.391	NS
B	1	267.3	0.37	267.3	267.3	0.99	0.341	NS
C	1	301.6	0.41	301.6	301.6	1.12	0.313	NS
D	1	2395.8	3.28	2395.8	2395.8	8.89	0.012	S
E	1	1611.5	2.2	1611.5	1611.5	5.98	0.032	S
Square	5	53958.5	73.77	53958.5	10791.7	40.07	0	S
A^2	1	20884	28.55	25489.5	25489.5	94.64	0	S
B^2	1	4699.7	6.43	6426.6	6426.6	23.86	0	S
C^2	1	16785.4	22.95	17360	17360	64.45	0	S
D^2	1	9000.3	12.3	8154	8154	30.27	0	S
E^2	1	2589.2	3.54	2589.2	2589.2	9.61	0.01	S
2-Way Interaction	10	11434	15.63	11434	11434	4.25	0.013	S
A x B	1	1295.1	1.77	1295.1	1295.1	4.81	0.051	NS
A x C	1	6078.9	8.31	6078.9	6078.9	22.57	0.001	S
A x D	1	321.5	0.44	321.5	321.5	1.19	0.298	NS
A x E	1	1090.8	1.49	1090.8	1090.8	4.05	0.069	NS
B x C	1	677	0.93	677	677	2.51	0.141	NS
B x D	1	11.1	0.02	11.1	11.1	0.04	0.843	NS
B x E	1	981.1	1.34	981.1	981.1	3.64	0.083	NS
C x D	1	510.7	0.7	510.7	510.7	1.9	0.196	NS
C x E	1	392.9	0.54	392.9	392.9	1.46	0.252	NS
D x E	1	74.8	0.1	74.8	74.8	0.28	0.609	NS
Error	11	2962.8	4.05	2962.8	269.8			
Lack of Fit	6	2739.9	3.75	2739.9	456.7	10.25	0.011	
Pure Error	5	222.8	0.3	222.8	44.6			
Total	31	73146	100					
Where A: Induction temperature, °C; B: Agitation rate, rpm; C: Induction time, h; D: Glucose concentration, %(w/v); E: Lactose concentration, % (w/v)								
S, Significant; and NS, Not significant								
R^2 = 95.95%; Adj R^2 = 88.58%; and Predicted R^2 = 0.00%								

Further, to increase the predictability of the model the most insignificant factors (A, B, C, AD, BC, BD, CD, CE, and DE) were removed from Equation 2 and the final modified regression model was obtained for the total activity of recombinant chitin deacetylase is shown below:

$$Z_2 \text{ (Total activity, U/L)} = 121.81 + 9.99\ D + 8.19\ E - 29.48\ A^2 - 14.80\ B^2 - 24.33\ C^2 - 16.67\ D^2 + 9.40\ E^2 + 9.00\ A \times B - 19.49\ A \times C - 8.26\ A \times E - 7.83\ B \times E. \quad (3)$$

The predicted values of the total activity were given by the software based on a modified regression model (Equation 3) and are represented in Table 5. In addition, the normality assumption is fulfilled as the residuals are normally distributed i.e., the data points are closer to the straight line as shown in normal probability plot (Fig. 2) indicating the capability of the model to optimize the expression of recombinant chitin deacetylase. Hence the modified model Equation 3 shall be applied to discover the optimal levels and their design space for the process.

Table 5
Analysis of variance (ANOVA) for the reduced model, test of significance for the expression of recombinant chitin deacetylase in *E. coli* Rosetta pLysS cells.

Source	DF	Seq SS	Contribution, %	Adj SS	Adj MS	F-value	P-value	
Model	11	67411.7	92.16	67411.7	6128.3	21.37	0.009	S
Linear	2	4007.2	5.48	4007.2	2003.6	6.99	0.028	S
D	1	2395.8	3.28	2395.8	2395.8	37.64	0	S
E	1	1611.5	2.2	1611.5	1611.5	5.62	0.028	S
Square	5	53958.5	73.77	53958.5	10791.7	37.64	0	S
A^2	1	20884	28.55	25489.5	25489.5	88.9	0	S
B^2	1	4699.7	6.43	6426.6	6426.6	22.41	0	S
C^2	1	16785.4	22.95	17360	17360	60.55	0	S
D^2	1	9000.3	12.3	8154	8154	28.44	0	S
E^2	1	2589.2	3.54	2589.2	2589.2	9.03	0.007	S
2-Way Interaction	4	9446	12.91	9446	2361.5	8.24	0	S
A x B	1	1295.1	1.77	1295.1	1295.1	4.52	0.046	S
A x C	1	6078.9	8.31	6078.9	6078.9	21.2	0	S
A x E	1	1090.8	1.49	1090.8	1090.8	3.8	0.065	NS
B x E	1	981.1	1.34	981.1	981.1	3.42	0.079	NS
Error	20	5734.3	7.84	5734.3	286.7			
Lack of Fit	15	5511.5	7.35	5511.5	367.4	8.24	0.14	NS
Pure Error	5	222.8	0.3	222.8	44.6			
Total	31	73146	100					

Where A: Induction temperature, °C; B: Agitation rate, rpm; C: Induction time, h; D: Glucose concentration, %(w/v); E: Lactose concentration, % (w/v).

S, Significant; and NS, Not significant.

R^2 = 92.16%; Adj R^2 = 87.85%; and Predicted R^2 = 72.83%.

The impact of interactions among the independent factors was visualized by the two-dimensional contour plots for the expression of recombinant chitin deacetylase (Fig. 3).

Validation of model

The response optimizer tool in MINITAB 17.0 (Trial version) was used to solve the reduced regression model (Equation 3) and to find the optimal conditions for enhanced total activity of recombinant enzyme chitin deacetylase and their expression in *E. coli* Rosetta pLysS. The model (Fig. 3) was validated at the optimal process conditions i.e., induction temperature of 22°C; agitation rate, 128 rpm; induction time, 30 h; glucose concentration, 0.058% (w/v); and lactose concentration, 1% (w/v). The optimal values (Table 6 and Fig. 4) of all the five factors except factor E (lactose concentration) are placed within factor levels selected and the predicted and experimental total activity of recombinant chitin deacetylase at these optimal conditions was 190.85 U/L and 202.39 ± 0.31 respectively (Table 6).

Table 6
The optimum process conditions for increased total activity of recombinant enzyme chitin deacetylase and their expression in *E. coli* Rosetta pLysS.

Parameters	Symbol	Optimal values	Total Activity, U/L	Total Activity, U/L
			Predicted	Experimental
Induction Temperature, °C	A	22	190.85	202.39 ± 0.31
Agitation rate, rpm	B	128		
Induction time, h	C	30		
Glucose concentration, % (w/v)	D	0.058		
Lactose concentration, % (w/v)	E	1.0		
The experiment was performed in triplicates and the value was represented in average with standard deviation.				

The optimal value of lactose concentration using the response optimizer tool was found to be 1% which was at a +2 level in the model. Therefore, the impact of higher lactose concentrations (1 – 2.5%(w/v)) on expression and total enzyme activity was studied. The total enzyme activity was 201.840±1.92 U/L, 201.900±1.95 U/L, 202.186±1.59 U/L and 202.173±2.09 U/L with 1%, 1.5%, 2% and 2.5% lactose respectively. There was no significant difference in the total activity and the SDS-PAGE analysis showed the expression level was the same in all lactose concentrations (Fig. 5).

Fermentation kinetics of lactose induction

To investigate lactose induction, expression profiling was done. The biomass, glucose concentration in the media, and enzyme activity were determined during the expression at the optimized conditions. The growth of *E. coli* showed a diauxic pattern due to the inclusion of glucose and lactose in the media. The first log phase was observed till 12 h at which the glucose was completely consumed. The expression of CDA and production of by-product allo-lactose were initiated in the second log phase after the 4 h of lag i.e., at 16th h. The maximum biomass yield and CDA activity of 17.53 ± 0.07 g/L and 202.39 ± 0.31 U/L respectively were found at 30 h of fermentation (Fig. 6).

Discussion

Chitin, the second most abundant polymer after cellulose is extracted on an industrial scale from the seafood waste (Yadav et al. 2019). The commercial applicability of chitin is restricted due to its crystalline structure. This limitation is addressed by deacetylating chitin into chitosan which increases its amorphous nature (Jayakumar et al. 2010). On a commercial scale, a chemical route is undertaken. However, the reproducibility of the product is a major concern in addition to other environmental apprehensions. The greener route using enzymes is thus being exploited. As the main source of chitin is from the sea, the unexplored marine ecosystem holds a plethora of enzymes with unique physiochemical properties. This has led to increased research for novel chitin modifying enzymes, chitin deacetylase (CDA) being one of them (Pawaskar et al. 2019). The array of CDAs can be employed to create chitosan with a unique pattern of deacetylation which would increase their biomedical applications (Cord-Landwehr et al. 2020; Wattjes et al. 2020). In the present study, fifteen halophilic microorganisms were isolated from the marine sediment. The primary screening resulted in four positive isolates. In the quantitative spectrophotometric analysis, isolate MS7 had the maximum CDA activity. The genetic identification of isolate MS7 showed a 99% match with *Bacillus aryabhattai* B8W22 (Pawaskar et al. 2021). The CDA gene was identified based on the homology-based comparison with *Bacillus megaterium* as the reference organism. The next step in the production workflow is to increase the yield of the expressed protein. *Escherichia coli* is the organism of choice due to its ease of cultivation and cost-effectiveness of the process (Rosano and Ceccarelli 2014). In the present study, the *Ba*CDA gene (~765 bp) was amplified and expressed in *E. coli* Rosetta pLysS cells. The heterologous overexpressed gene product of *Ba*CDA had a molecular weight of 29 kDa. The size was within the range of the reported CDA from various sources (25-80 kDa) (Kaczmarek et al. 2019). The production of proteins in the *E. coli* through an efficacious platform with high growth yield but suffers from soluble expression bottlenecks requiring process and media optimization. The growth medium optimization with one factor at a time (OFAT) is tedious and lengthy approach (Mandenius and Brundin 2008; Papaneophytou and Kontopidis 2014; Gutiérrez-González et al. 2019). Hence a statistically designed experiment approach to screen the process and media parameters to optimize the recombinant CDA production in the which is missing in the literature was explored. The reports on the usage of CCD for optimization of the CDA production in the wild strains of *Aspergillus flavus*, *Penicillium oxalicum* and bacterial CDA from *Bacillus amyloliquefaciens* (Pareek et al. 2011; He et al. 2014; Yonis et al. 2019) are reported. To optimize the expression in the *E. coli* system, different parameters were taken into consideration. The induction temperature is a very important parameter as the variations in the temperature lead to the expression of the enzyme in either soluble form or as an inclusion body (Rosano and Ceccarelli 2014). In the present study too, the fermentation at 16 °C led to increased expression of the CDA as a soluble fraction in contrast to the fast growth rate at 37 °C. The slower growth at 16 °C gave *E. coli* adequate time for the CDA protein folding post expression (Table 3 and Fig. 1). A similar trend was observed with the agitation speed which improves the oxygen transfer rate and thereby the growth of the *E.coli* (Rosano and Ceccarelli 2014). The agitation speed also affects the growth of *E. coli* during expression. Therefore, an accord was maintained at 128rpm which was optimum for growth and soluble expression of *Ba*CDA, a further increase led to negative effects on the expression. A similar observation with the agitation rate has been reported by Shahzadi et al. in their study wherein an increase of agitation was causing downregulation of recombinant gene expression.(Shahzadi et al. 2021). The pET expression system in conjunction with the *E. coli* as the host organism is used for high productivity. The heterologous protein is induced by the inclusion of IPTG, however, research groups have opted for lactose as an inducer to create auto-induction conditions and to reduce the cost of the product as lactose is a cheaper substitute for IPTG (Wurm et al. 2016). Hence the expression of the recombinant enzymes in the *E. coli* system was dependent on the glucose-lactose diauxic (Mostovenko et al. 2011). Therefore, based on the literature and our preliminary experiments, the process parameters (induction temperature, agitation rate, induction time, glucose concentration, and lactose concentration) were selected for *Ba*CDA expression in *E. coli* with five different levels using a central composite design (Table 2). The total activity

varied from 1.27 to 199.72 U/L indicating the influence of factors and their levels on the expression of *Ba*CDA in *E. coli* Rosetta pLysS cells (Table 2). A variation of only ± 9.71% was observed in the total activity between experimental and predicted values (Table 2) indicating the accuracy of experimentation.

The adequacy of the model and fitness was evaluated by using analysis of variance (ANOVA) for the experimental design used (Table 5). The high value of R^2 suggests a higher significance of the model (Selvaraj et al. 2021). The observed low difference of 0.431 between the adjusted R^2 (0.8785) and the R^2 value (0.9216) confirms the data accuracy. The model Equation 2 was highly significant with an F-value of 21.37 as shown by Fisher's F-test, along with a very low probability value (P model > F=0.000), which was significant at a 95% confidence interval. The model F-value was calculated using the formula:

$$F-value = \frac{Meansquareofindividualterm}{Meansquareofresidual}$$

The factors are said to be significant only if the value of F-statistics probability is less than 0.05. For the proposed model (Equation 3), the terms D, E, A^2, B^2, C^2, D^2, E^2, AB, and AC were significant at 95% confidence ($P < 0.05$) (Table 5). The value of “Lack of Fit F-value” was 8.24 which indicates its insignificance and has a 14% chance to be significant (Table 5) The insignificant F-value of “Lack of Fit” represents the fitness of experimental data to the model. Therefore, based on the above statistics it can be concluded that glucose concentration (D) and lactose concentration (E) exhibits a vital part in the expression of recombinant chitin deacetylase in *E. coli*. In this study, the physical environmental conditions such as induction temperature (A), agitation rate (B), and induction time (C) did not show significance in the expression of recombinant chitin deacetylase (Table 5). Whereas the interaction between A and B; and A and C was very much significant (Table 5). The results are in good agreement with the general facts of higher F-values of the model than the F-value of lack of fit, and higher R^2 values (> 0.70) specify that the model fits the data in a better way. Further, to validate the polynomial regression model (Equation 3), experiments were carried out at the optimal conditions in triplicates as given in Table 6 and the experimental value was found to be 202.39 ± 0.31 U/L. The good correlation between the values of predicted and experimental total activities are very much closer validating the competence of the model. This statistical optimization technique enhanced the total activity and expression of recombinant enzyme chitin deacetylase in *E. coli* from 84.67±0.56 U/L (unoptimized condition) to 202.39 ± 0.31 U/L (optimized condition), a ~2.39-fold increase.

The kinetics of the expression profiling was carried out with the optimized process conditions. The glucose concentration, biomass yield, and total enzyme activity were evaluated. As glucose is the simplest form of carbon, for the initial growth *E. coli* utilizes glucose first. Once the glucose exhausts in media, the *E. coli* starts utilizing lactose. In this study, glucose (0.061%) was exhausted in 16 h of fermentation, leading to the onset of the diauxic shift. There was a second lag phase during this sugar utilization transition lasting for 4h. The expression of the *Ba*CDA increased strongly during this diauxic shift. The culture reached its saturation at 30 h recording maxima in the total enzyme activity (202.39±0.31 U) and biomass (17.53±0.07 g/L). The total activity after the optimization had increased by a fold of ~2.39 fold. The total fermentation duration had also reduced from the earlier recorded 52 h to 30 h proving it be better on economics. Many groups have worked on the process optimization with lactose as the inducer using CCD as the statistical tool (Table 7). The fold improvement after the statistical intervention varied from 2.1 folds in lipase (Ghahremanifard et al. 2018),16.5 folds in sucrase (Vuillemin et al. 2014), 3 folds with the study on Ficin (Sattari et al. 2020). In the same lines, we could upregulate the production of *Ba*CDA by ~2.39 folds. In the present study, we have worked on the diauxic of *E. coli* rather than adding it as an inducer based on the absorbance value at 600nm. This auto-induction aspect helps to reduce the chances of contamination required during regular absorbance monitoring Even though lactose was used as an inducer, most of the studies optimized for the OD_{600} for induction (Table 7). On the other hand, in this study, we choose to auto-induce by including lactose in the media along with glucose as a repressor.

Table 7
Literature on lactose-induced expression optimization using the CCD model.

Sl. No.	Protein	Vector	Host	Factors	Media	Lactose concentration	Post-induction temp	Post-induction time	Improvement	Reference
1	Lipase	pET-26b	*E. coli* BL21 (DE3)	Lactose concentration, Induction time and Temperature	LB	12.5% (w/v)	24°C	15 h	mSpA increased CALB expression levels by 2.1-fold relative to the control	(Ghahremanifard et al. 2018)
2	Activin A	pET-21a	*E. coli* (BL21(DE3) and BL21(DE3) plysS)	IPTG concentration and Lactose concentration (CCD), Post-induction time and Temperature (one variable at a time)	SOB	0 (IPTG at 1.5mM concentration induced at 0.6 OD_{600})	30°C and 35°C	10 h and 4 h	-	(Hajihassan and Biroonro 2018)
3	α-(1→2) branching sucrase	pET-55	*E. coli* BL21 Star DE3	Culture duration, Temperature, Glycerol, Lactose and Glucose	LB	1% (w/v)	23°C	26.5 h	The 165-fold increase compared to the production levels previously reported.	(Vuillemin et al. 2014)
4	Staphylococcal protein A (SpA)	pET-26b	*E. coli* BL21 (DE3)	Lactose, Glycine, Induction time, Optical density (600) and Temperature	LB	10% (w/v) [at OD 1.1]	33˚C	11 h	A 5-fold increase in the secretion of SpA increased	(Rigi et al. 2014)
5	Ficin	pET-28b	*E. coli* BL21 (DE3)	Lactose concentration, Bacterial OD at induction time, Temperature, Incubation time after induction and Shaking rate	LB	10% [at OD 1.25]	24°C [115 rpm]	7 h	The optimization procedures increased the amounts of ficin production by approximately 3 folds	(Sattari et al. 2020)
6	Chitin deacetylase	pET-22b	*E. coli* Rosetta pLysS	Induction temperature, Induction time, Agitation rate, Lactose concentration and Glucose concentration	TB	1% (w/v)	20°C [128rpm]	14 h	~2.39-fold increase in the total enzyme activity	Present study

LB - Luria Broth, SOB - Super Optimal Broth, IPTG - Isopropylthio-β-galactoside, *E. coli* - Escherichia coli

Conclusion

The Central composite design was useful for modelling the effects of bioprocess and nutritional parameters on the CDA production by *E.coli* Rosetta pLysS cells in submerged fermentation. This also enabled us to identify the optimal conditions for the CDA expression. The optimized conditions for the submerged fermentation included induction temperature of 22°C, agitation speed of 128 rpm with incubation for 30 h without pH control. The media included 0.058% glucose and 1% lactose. The optimized parameters were validated by comparing the theoretical value with the experimental responses. At optimized conditions, the total enzyme activity yield was enhanced by ~2.39 folds and the fermentation duration was reduced to 30 h from 52 hours.

The investigation of induction kinetics showed the expression of *Ba*CDA initiates after 16th h on utilization of lactose as a carbon source by *E. coli* cells. Therefore, the post-induction duration was only 14 h whereas in the unoptimized condition it was 28 h. The findings could be used in scaling up the expression of *Ba*CDA with minimum culturing time to get a high yield thereby making the fermentation process cost-effective. Therefore, the optimized process conditions could be scaled up for higher yield of CDA which can be utilized for the pharmaceutical grade chitosan production.

Abbreviations

ANOVA
Analysis of variance
BLAST
Basic Local Alignment Search Tool
CDA
Chitin deacetylases
CE-4
Carbohydrate esterase family 4
CAZY
Carbohydrate-Active enZYmes
CCD
Central composite design
DNA
Deoxyribonucleic acid
E. coli
Escherichia coli
EGC
Ethylene glycol chitin
IPTG
Isopropylthio-β-galactoside
LB
Luria-Bertani
NCBI
National Center for Biotechnology Information
OFAT
One factor at a time method
PCR
Polymerase chain reaction
RSM
Response surface methodology
SDS-PAGE
Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
TB
Terrific broth

Declarations

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All the authors consented to the publication of this work.

Conflicts of interest

The authors declare that they have no competing interests.

Availability of data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author's contribution

GMP has performed, analyzed, and written the manuscript. RR has conceptualized, supervised, investigated, administrated the project, and edited the manuscript. SBL has computed, analyzed, investigated, reviewed, and edited the manuscript. All authors have read and approved the final manuscript.

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Figures

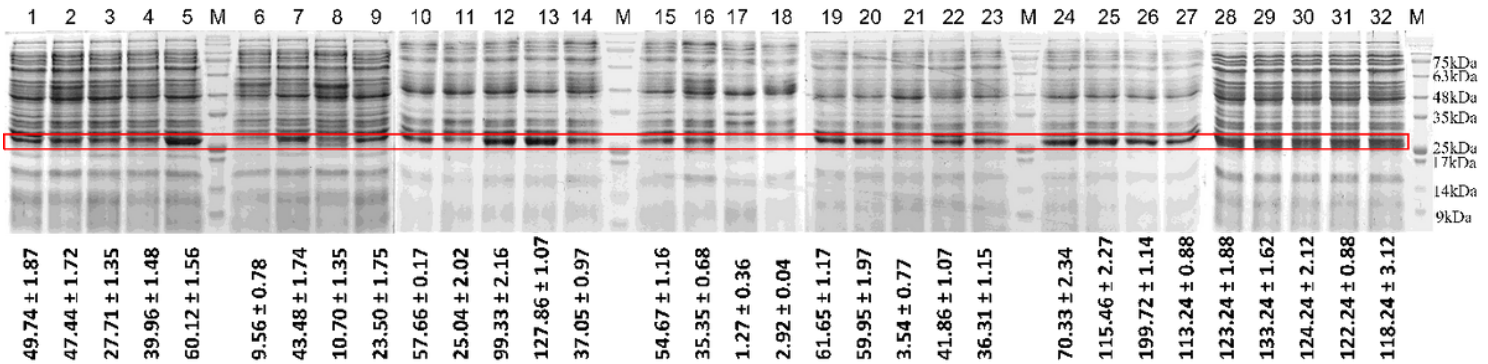

Figure 1

SDS-PAGE analysis on the expression of extracellular recombinant enzyme chitin deacetylase in *E. coli* Rosetta pLysS for all the experimental runs given by central composite design. The expression of CDA was observed at 29 kDa.

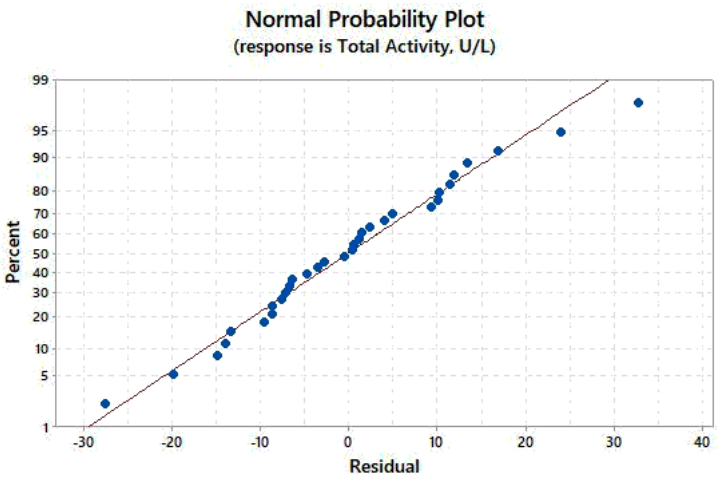

Figure 2

The normal probability plot. The plot indicates the capability of the model to optimize the expression of recombinant chitin deacetylase.

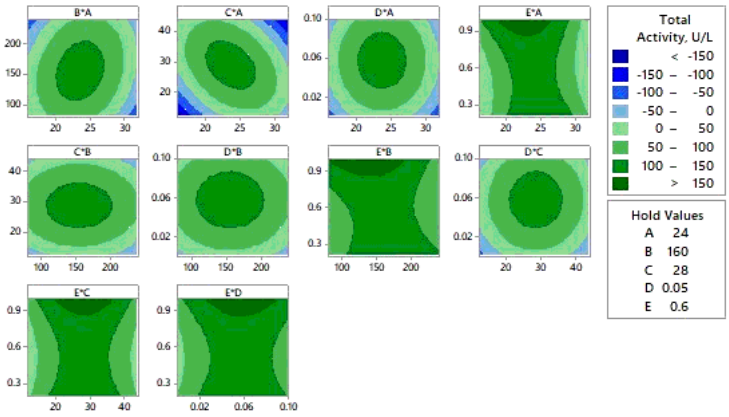

Figure 3

The contour plots of all the combinations of factors. A: Induction temperature, °C; B: Agitation rate, rpm; C: Induction time, h; D: Glucose concentration, %(w/v); and E: Lactose concentration, % (w/v).

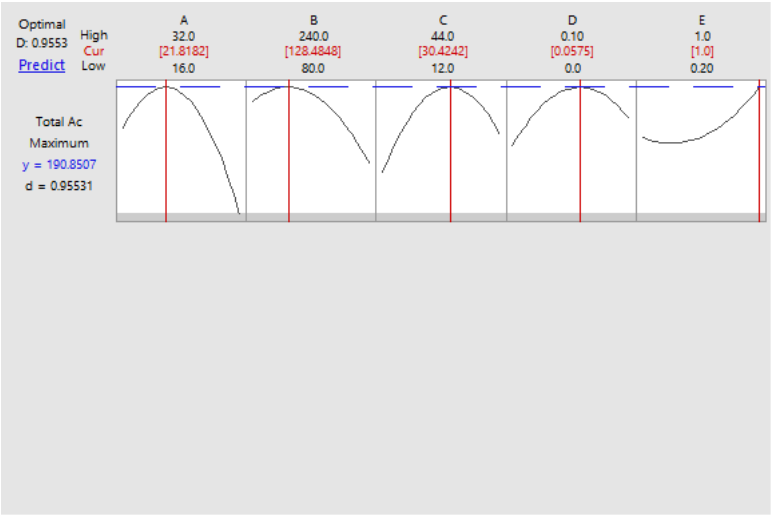

Figure 4

The optimization plot presenting optimal values for the increased total activity of recombinant enzyme chitin deacetylase and their expression in *E. coli* Rosetta pLysS.

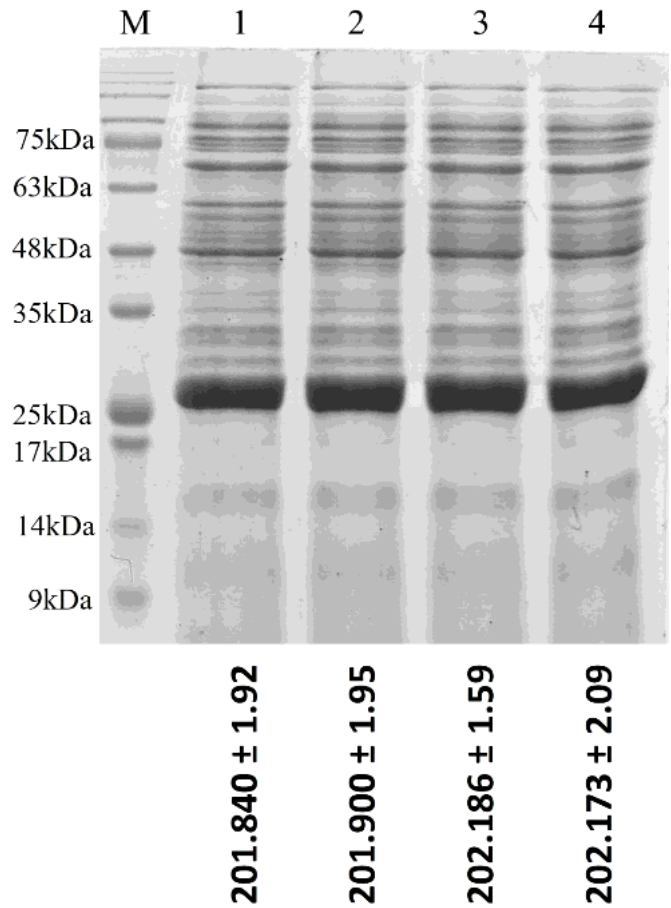

Figure 5

SDS-PAGE analysis on the expression of extracellular recombinant enzyme chitin deacetylase in *E. coli* Rosetta pLysS with different lactose concentrations in an optimized process condition.

Lane 1: Marker; Lane 2: 1 % (w/v) lactose concentration; Lane 3: 1.5 % (w/v) lactose concentration; Lane 4: 2 % (w/v) lactose concentration; and Lane 5: 2.5 % (w/v) lactose concentration.

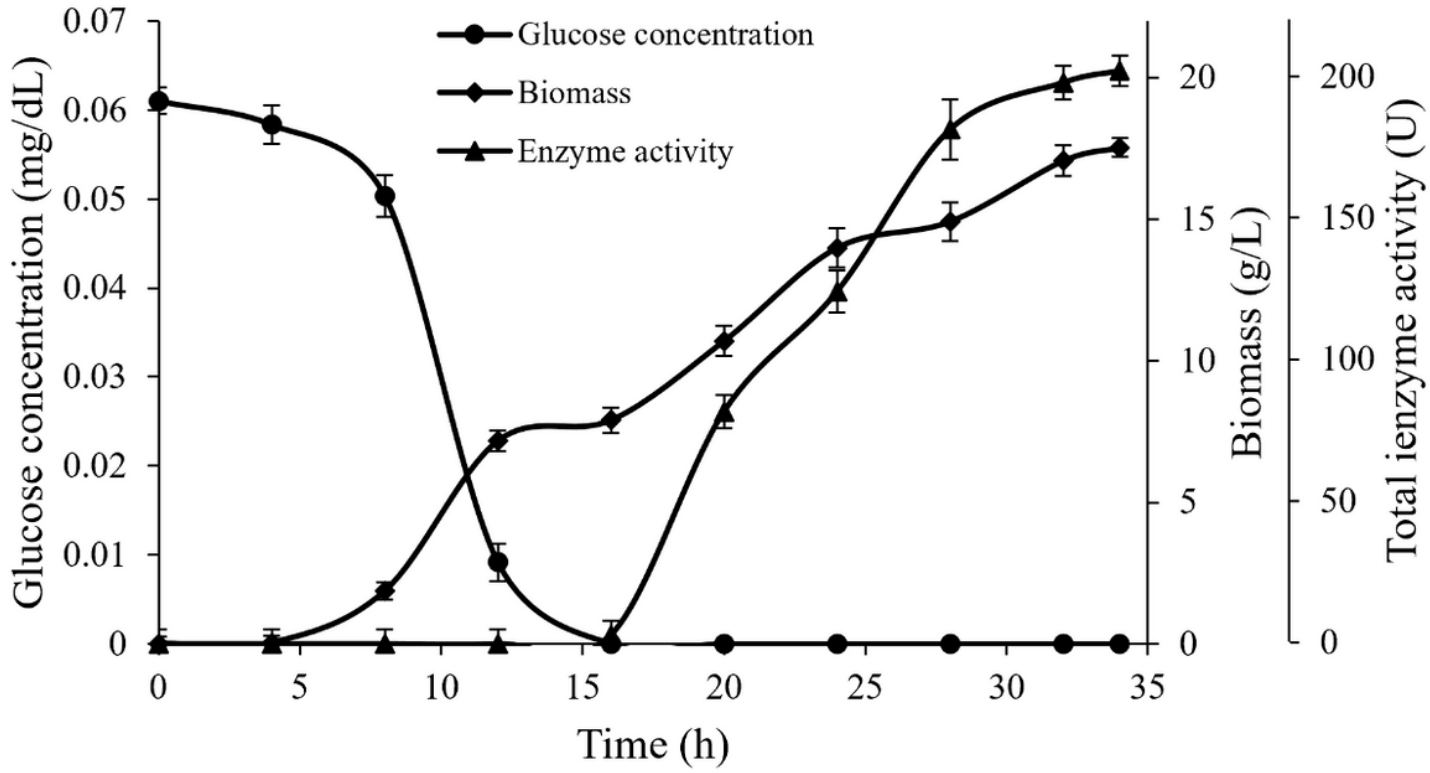

Figure 6

Kinetics of biomass yield, glucose concentration, and total enzyme activity in expression of extracellular recombinant enzyme chitin deacetylase in *E. coli* Rosetta pLysS at the optimized process conditions.

Supplementary Files

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- 2Graphicalabstract.docx