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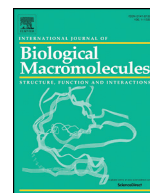
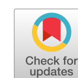


Expression of *Bacillus licheniformis* chitin deacetylase in *E. coli* pLysS: Sustainable production, purification and characterisation



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ABSTRACT

Chitosan obtained by enzymatic deacetylation of chitin using chitin deacetylase (CDA) holds promise primarily due to the possibility to yield chitosan with non-random patterns of acetylation and more environmentally friendly process compared to chemical deacetylation. In the present study, a sustainable bioprocess is reported for over-expression of a bacterial CDA in *E. coli* pLysS cells. A *Bacillus licheniformis* CDA gene is identified in the genome of the bacterium, cloned, and expressed, yielding enzymatically active recombinant protein. For enzyme production, a growth medium is formulated using carbon and nitrogen sources, which do not compete with the human food chain. The maximum enzyme activity of 320 ± 20 U/mL is achieved under optimized conditions. The CDA productivity is improved by about 23 times in shake flask culture by optimizing operating conditions and medium components. The CDA is purified and the enzyme kinetic values i.e. K_m, V_{max} and K_{cat} are reported. Also the effect of cofactors, temperature, and pH on the enzyme activity is reported. Further, economic yield is proposed for production of CDA through this bioprocess.

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1. Introduction

Biopolymers like cellulose, lignin, chitin etc. are attaining importance in the number of industrial sectors and their production and application is the main focus of present day biotechnology. Recently commercial potential of chitin and its soluble derivative chitosan has been explored [1–3]. Chitosan the deacetylated, non-toxic, biodegradable derivative of chitin, is soluble in acidic solutions, and a much more tractable material than chitin. Based on these properties chitosan has wide application in various fields like water treatment, cosmetics, agriculture, pharmaceuticals, food industry and biomedicine [4]. Currently chitin is converted to chitosan using chemical or bio-catalytic alkaline deacetylation process having limitations like high water footprint, environmental pollution due to effluent generated from concentrated alkaline solution and broad and heterogeneous range of soluble and insoluble products [4]. Chitin deacetylases (CDA) are type of hydrolases which convert chitin into chitosan by deacetylation of the amino group of chitin. CDA gained much interest in recent decade because of its high degree of specificity in conversion of the second most abundant but hydrophobic and crystalline biopolymer chitin to one of the very promising cationic biopolymer chitosan [5,6]. Tsigos et al., provided a critical review on CDAs, in which they discussed about properties, applications characterization of this enzyme [7]. CDA was first identified and partially purified from the mycelial extracts of *Mucor rouxii* [8]. Since then various CDA sources were identified and reported [7,9,10]. In the past few years, numerous microorganisms with CDA activity have been explored but most of them are intracellular CDA producers with limited yield [9,11–14]. The chitin based product applications expanded to biosynthesis of even more versatile chito-oligosaccharides [15]. For industrial application, it is important to explore the novel CDA hyper producers. As, a result, the emphasis in the CDA research extended to identify novel CDAs [16], in silico characterization [17], cloning and expression [16,18,19]. Although there is much research on cloning and expression of CDA for various applications, the literature in bioprocess development and optimization of CDA is scarce [10,20,21]. Optimization of processing parameters play an important role in the development of any fermentation process owing to their impact on the economy and efficacy of the process. The conventional approach of optimizing parameters one-factor-at-a-time provide a systematic basis for further optimization. The present study reported the development of a bioprocess for CDA (from *Bacillus licheniformis*) production in recombinant *E. coli* pLysS, which improved productivity of the CDA biosynthesis by 23 folds.

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2. Materials and methods

2.1. Cloning of CDA gene into E. coli pLysS

The CDA gene of *Bacillus licheniformis* was obtained from the metagenomic library (Gene bank accession number PRJEB6317) of the soil exposed to chitin [22]. The gene cloning procedure and vector map is reported by Raval et al. [23].

2.2. Expression of CDA in E. coli Rosetta pLysS

A starter culture was prepared in LB broth (Tryptone 10 g/L, Yeast extract 5 g/L and NaCl 10 g/L) inoculated with loop full of bacteria from the culture plate, which was supplemented with ampicillin and chloramphenicol having concentration 100 μg/mL and 35 μg/mL respectively. It was kept in incubator shaker at 37 °C until the O.D of the culture reached 0.6–0.7. A 250 mL shake flask with a medium volume of 80 mL was inoculated with 5% inoculum from the starter culture. The culture was supplemented with antibiotics, induced by 1 mM IPTG at O.D. of 0.6 to 0.7, and incubated at 37 °C at 120 rpm. Samples were taken at regular time intervals for the analysis of biomass and CDA activity. The correlation between $O.D_{600}$ and biomass was obtained as, Biomass (g/L) $= 0.503 \cdot O.D_{600}$.

2.3. Study of enzyme (CDA) activity

The 3-methyl-2-benzothiazoline hydrazone (MBTH) assay was used to study the enzyme activity [24]. The broth was centrifuged at 10,000 rpm for 15 min at 4 °C. The pellet obtained was mixed with 1 mL of Tris-HCl buffer of pH 6.5. The cells lysis was done using the probe type sonicator (130 W). Pulse of 5 s on and 1 s off was used for 5 min with 20% amplitude. The cell lysate was centrifuged at 10,000 rpm for 15 min at 4 °C, the supernatant was filtered through a 0.45 μm syringe filter and the filtrate was diluted 10 times to analyze the intracellular enzyme activity. 250 μL of glycol chitin (1 mg/mL), 250 μL of Tris-HCl buffer (pH 6.5) and 100 μL of the diluted supernatant was added to the assay mixture as a source of the enzyme. This mixture was incubated at 37 °C with shaking for 1 h. 500 μL of 5% $KHSO_4$ was added to stop the reaction and kept for 5 min. 500 μL of 5% $NaNO_2$ was added to the samples. Addition of $NaNO_2$ was followed by 6 h of incubation in the fume hood to release all the NO_2 gas. This was followed by the addition of 0.5 mL of ammonium sulphamate (12% by wt.) and 0.5 mL of 0.5% MBTH and kept for 1-hour incubation. Finally, 0.5 mL of 0.5% $FeCl_3$ prepared in 0.1 N HCl was added. The reaction mix was incubated for 1-hour and the absorbance was measured at O.D 656 nm. One Unit of the CDA activity is defined as the activity, which released 1 μmol of glucosamine from the Glycol chitin per minute. The enzyme activity of the sample was calculated accordingly using MBTH assay as well as with enzymatic acetate assay kit (Sigma Aldrich, Catalog no. MAK086).

2.4. Effect of rotational speed

The 250 mL conical flasks with 80 mL medium volume were incubated in orbital shaker at 120, 200 and 250 rpm to investigate the effect of rotational speed on biomass and CDA activity.

2.5. Effect of medium filling volume on biomass growth and CDA production

The 250 mL conical flasks were filled with different amounts of medium volumes to study the effect of medium volumes on biomass and enzyme activity. The flasks were operated at 250 rpm and 37 °C in an orbital shaker.

2.6. Study of the CDA production in different media

The CDA production was studied in three different media namely Luria Bertani Broth (Tryptone 10 g/L, Yeast extract 5 g/L and NaCl 10 g/L), Nutrient Broth (Peptic digest of animal 5 g/L, NaCl 5 g/L, Beef extract 1.5 g/L and yeast extract 1.5 g/L) and Tryptic Soy Broth (Pancreatic digest of casein 17 g/L, NaCl 5 g/L, paptic digest of soya bean meal 3 g/L, Di-potassium hydrogen phosphate 2.5 g/L and Dextrose 2.5 g/L). The flasks were filled with 12 mL medium volume and operated at 250 rpm and 37 °C for 8 h.

2.7. Study of the effect of different nitrogen sources on CDA production

The organic nitrogen sources such as yeast extract, beef extract, bacteriological peptone, and tryptone were added at 10 g/L concentration to the LB broth. The flasks were filled with 12 mL medium volume and operated at 250 rpm and 37 °C for 8 h.

2.8. Purification by Ni-NTA chromatography

The expressed CDA was purified using the Ni-NTA matrix (Qiagen, Germany). The column was equilibrated with 10 mL of lysis buffer. Lysate was passed through column 4 times by gravity flow. The column was washed with 10 mL of 100 mM wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 100 mM Imidazole, 10% glycerol). Protein was eluted with 250 elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, 10% glycerol) followed by 500 elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM Imidazole, 10% glycerol). All elution samples were collected and subjected to SDS-PAGE.

2.9. Estimation of production cost

The raw material cost was calculated as,

$$C_T = \sum_{i=1}^{n} c_i y_i \qquad (1)$$

C_T = Total raw material cost in USD
c_i = Unit cost of raw material "i" in USD
y_i = Quantity of raw material used "i" in bioprocess

According to Bailey and Ollis, the raw material cost is the 30% to 40% of the total production cost, also the overhead charges and the labour cost are 15% each of the total production cost, therefore,

Total cost = Production cost + overhead charges + labour cost.

3. Results

3.1. Effect of operating condition on CDA production

Fig. 1 shows effect of rotational speed and filling volume on the growth and CDA production in recombinant *E. coli* pLysS cells. As rotational speed increased (Fig. 1, solid lines), the growth rate improved in the LB medium due to which the time required to attain the maximum biomass reduced. The culture grown at 250 rpm reached the maximum biomass of about 1 g/L in about 30 to 34 h, whereas the culture grown at 120 rpm achieved maximum biomass at about 60 h. Further, reduction in the medium filling volume in the flask reduced the time required to achieve maximum biomass. Fig. 1 demonstrates the effect of medium filling volume on the growth of the microbial culture (dashed lines). As filling volume reduced from 40 mL to 12 mL, the time required to reach the maximum biomass concentration of about 1 g/L reduced from about 30 to 34 h to only about 8 h. The oxygen transfer rate in

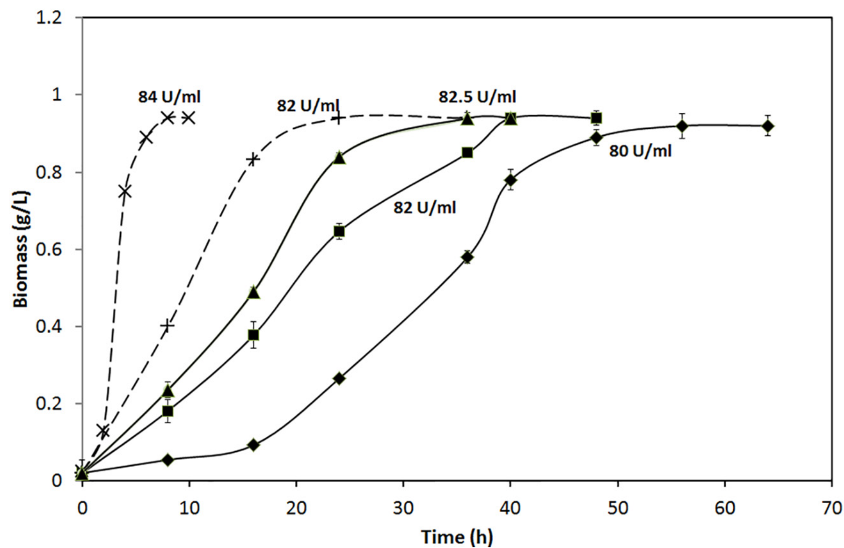

Fig. 1. Effect of rotational speed (solid line - (♦) 120, (■) 200, (▲) 250 rpm) and medium volume (dashed line - (+) 40 ml, (X) 12 ml) on recombinant *E. coli* growth in LB medium.

shake flask cultures is proportional to (rotational speed)$^{1.18}$ and (medium volume)$^{-0.74}$ [17]. Thus, increasing the rotational speed of the shaker and reducing the medium filling volume improved the oxygen transfer rate from the air to the medium. Higher oxygen transfer rate improved the growth and hence reduced the time required to reach the maximum biomass concentration. The maximum enzyme activity in the LB medium varied between about 80 and 84 U/mL in above study.

Supplementary Fig. 1 shows the effect of different types of media on CDA activity. The cultures grown in TSB medium exhibited the maximum CDA activity as compared to the other investigated media. TSB medium contains 17 g/L of complex nitrogen source and 2.5 g/L of glucose. Whereas, the other two media do not contain glucose. The amount of complex nitrogen source available in other two media is also less as compared to TSB medium. Therefore, TSB medium produced more biomass of about 3 g/L as compared to about 1 g/L biomass in LB and NB medium, respectively. However, the time required to achieve the maximum CDA activity increased from about 8 h in LB/NB medium to 15 h in TSB medium. The CDA expressing plasmid has *lac* operon for expression of CDA gene where IPTG acts as an inducer. According to Mondin et al., glucose concentration as low as 2 mM repress the *lac* operon which was the reason behind delayed expression of recombinant CDA in TSB medium [18]. Since, glucose was repressing the expression of the CDA, glycerol was chosen as the carbon source for medium development. Moreover, glycerol does not compete with the human food chain, which makes it an ideal carbon source for bioprocess development. The effect of the different nitrogen sources on the CDA production was investigated. Supplementary Fig. 2A and B demonstrates the effect of the different nitrogen sources and different glycerol concentrations on the CDA expression. Yeast extract and beef extract exhibited almost similar enzyme expression of about 160 U/mL, followed by peptone and tryptone. The enzyme activity almost doubled in cultures supplemented with the different nitrogen sources as compared to the control culture grown in LB medium. The addition of glycerol further improved enzyme production and hence activity. As glycerol supplementation increased in the medium, the enzyme activity improved. The crude filtered supernatant gave the maximum enzyme activity of about 320 U/mL and the specific activity of about 256 U/mg in 2% (w/V) glycerol. Higher concentrations (3%) of the glycerol resulted in similar CDA activity at 10 h and therefore were not used in further studies. Sodium ions at a concentration of 1 g/L improved CDA activity

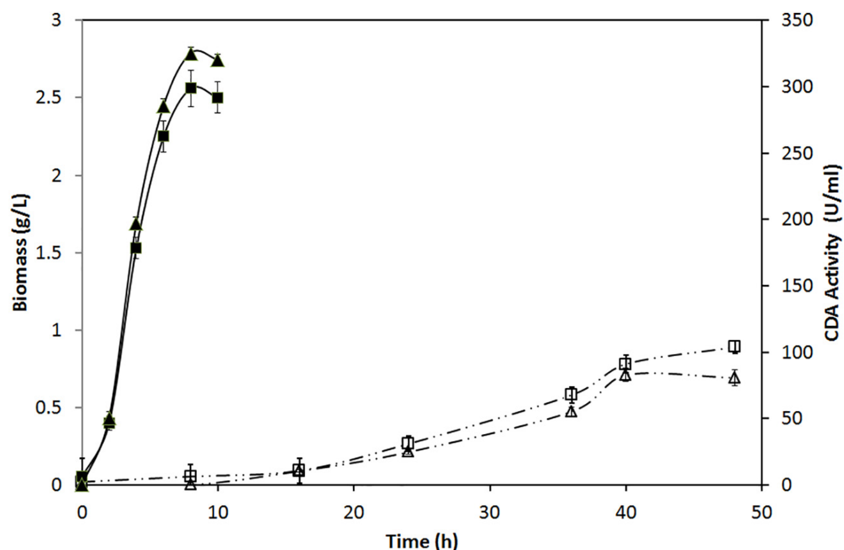

Fig. 2. Biomass growth (square symbol) and CDA activity (triangle symbol) in control conditions (□, △) and under optimized conditions (■, ▲).

Fig. 3. Effect of (A) Cofactors, (B) Cobalt concentration, (C) pH and (D) temperature on the purified CDA activity.

(data not shown). Fig. 2 compares the biomass and CDA activity in the control medium before optimization and after optimization of operating conditions and medium components. There was about 6-fold improvement in the CDA activity in optimized medium containing 2% glycerol, 10 g/L yeast extract and 1 g/L NaCl. Moreover, the bioprocess time reduced substantially from about 48 h to just 8 h. The increase in CDA activity and reduction in cultivation time increased volumetric productivity of CDA from 1.67 U/mL/h to 40 U/mL/h.

The CDA thus produced was purified by Ni-NTA matrix column (Qiagen, Germany). Supplementary Fig. 3 demonstrates the SDS-PAGE picture of the various stages of purification. The purified protein showed size of about 29 kDa, which is similar to the expected size. Fig. 3 shows the effect of the cofactors, temperature and pH on the specific activity. The maximum enzyme activity was observed at 50 °C and at 8 pH. Cobalt ions (8 mM) had the most pronounce effect on the enzyme activity. The purified enzyme activity was about 1400 U/mg under optimum

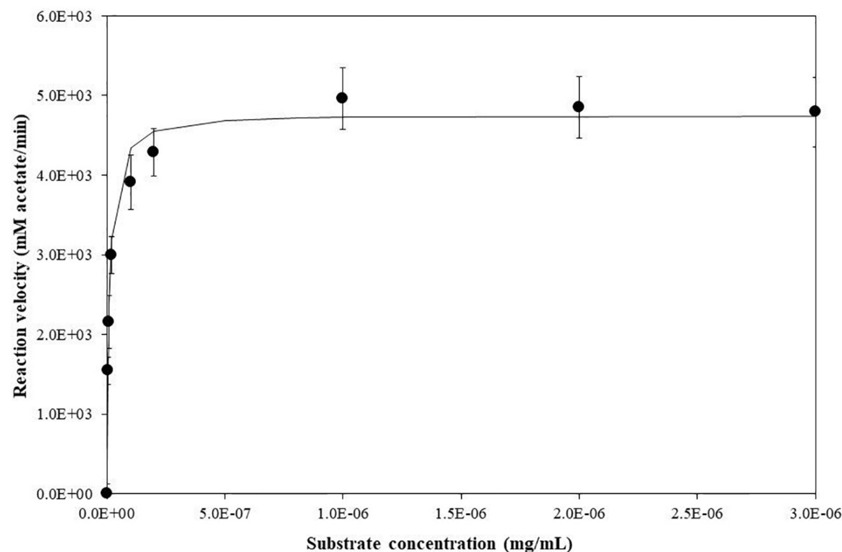

Fig. 4. The Michaelis-Menten plot for the enzymatic deacetylation of glycol chitin.

Table 1
The above calculations are based on 100 mg purified CDA obtained from 5 l of culture broth as per the method given in Biochemical Engineering Fundamentals by Bailey and Ollis [28], McGraw Hill.

Raw material For 100 mg purified CDA Cost obtained from alibaba.com	Unit cost USD (c_i)	Quantity (yi)	Cost USD ($c_i{}^*y_i$)
Yeast extract (per kg)	6	0.1 kg	0.6
Glycerol (per kg)	2	0.1 kg	0.2
Ni-NTA (per ml)	15	5 ml	75
Other chemicals (per kg)	20	0.1 kg	2
Total raw material (RM) cost $C_T = \sum_{i=1}^{n} c_i y_i$			77.8
Production cost (basis: 30% of production cost is RM cost)			259.3
Overheads (15% of production cost)			38.9
Labour cost (15% of production cost)			38.9
Total cost			337.1

conditions. Fig. 4 represents the relation between the reaction velocity with respect to substrate concentration. The data were fitted with non-linear regression using MS Excel solver and the K_m, V_{max} and K_{cat} values were obtained as 9.96E-09 mg/mL, 4.78E+03 mM acetate/min and 5.18E+03 1/s. respectively as given in Table 2.

4. Discussion

It is a common practice in biotechnology research labs to use 250 mL conical flasks, filled with about 50 to 80 mL of the liquid medium. The medium in the flasks is usually mixed in orbital shakers operated at 120 to 150 rpm. The growth rate of aerobic culture is dependent on the oxygen uptake rate of the culture. This uptake rate also depends on the oxygen transfer rate in the liquid medium from the air available in the headspace of the conical flask [25]. Meier et al., demonstrated that oxygen transfer rate in shaken cultures can be enhanced by various operating parameters such as, flask size, medium filling volume in the flask, rotational speed and shaking diameter of the shaker [26]. Thus, it is possible to increase growth rate of microorganisms by changing above mentioned parameters in the shaken cultures. This was demonstrated in Fig. 1. The addition of yeast extract and glycerol further enhanced the enzyme activity to about 320 ± 20 units/mL. The glycerol triggered glycolytic and gluconeogenic pathways for efficient utilization of carbon source in *E. coli* [27]. Martinez-Gomez et al. also observed enhancement in β-galactosidase activity in presence of glycerol [27]. In a bioprocess, the raw material cost share in the total production cost is usually in the range of 30 to 40%. The raw material cost for CDA production using the mentioned process is about 77.8 USD. If conservative estimate is taken and assume raw material cost is 30% of the production cost, then the production cost value is USD 259.3, which was calculate as total raw material cost/0.3. Adding labour cost and overhead charges (15% each of the total production cost), the total CDA production cost reaches to about 337 USD. Overall, the cost of production of 100 mg of purified CDA is mentioned in Table 1. The production cost is calculated based on the method given by Bailey and Ollis [28]. Out of the total cost of raw materials, more than 90% cost was attributed to downstream processing, i.e., purification of recombinant CDA using Ni-NTA matrix. Thus, developing a cost effective purification technique may reduce the cost of production further.

Table 2
Kinetic parameters of chitin deacetylase from *Bacillus licheniformis*.

Substrate	Kinetic parameters		
	K_m (mg/mL)	V_{max} (mM acetate/min)	K_{cat} (1/s)
Glycol chitin	9.96E-09	4.78E+03	5.18E+03

Researchers expressed and characterized novel recombinant CDA from various sources. Martinou et al., expressed chitin deacetylase gene (CDA2) from *Saccharomyces cerevisiae* in *Escherichia coli* and reported maximum enzyme activity of 30 U/L at pH 8 and 50 °C [29]. Chambon et al., expressed *Rhizobium NodB* chitin deacetylase in *E. coli* cultures in solubilized form and yield optimization gave up to 100 mg of purified deacetylase from 1 L of culture media [20]. Shreshtha et al., expressed chitin deacetylase from *Colletotrichum lindemuthianum* in *Pichia pastoris* [30]. They reported 72 U/mg activity at 60 °C and pH 8 in presence of cobalt cofactor and the yield was calculated approx. 0.26 g dry weight per gram methanol. Liu et al., identified a novel CDA like enzyme from metagenomic library of deep-sea sediments of arctic ocean [9]. They reported the maximum enzyme activity at pH 7.4 and 28 °C. Naqvi et al., reported a novel fungal CDA, which produced novel patterns of deacetylation [31]. Aranda-Martinez et al., expressed a novel CDA from the nematophagous fungus *Pochonia chlamydosporia with the overall yield of* 0.43 mg/L of culture media [32]. However, research reports on the bacterial CDA expression and characterization is scarce. Moreover, the bioprocess development for CDA production is in nascent stage and more efforts are needed to identify and produce novel CDAs for production of specific chitosan.

5. Conclusion

In the present work the recombinant CDA production and purification was optimized. The CDA production at optimal condition was 320 ± 20 U/mL in the culture broth. The purified CDA was characterized in terms of pH, temperature, co-factor and its concentration. The recombinant CDA showed the highest activity in the presence of cobalt ions at 8 mM concentration. The maximum activity was observed at pH 8 at an optimum temperature of 50 °C. The purified enzyme activity was about 1400 U/mg. The values of K_m, V_{max} *and* K_{cat} were obtained as 9.96E-09 mg/mL, 4.78E+03 mM acetate/min and 5.18E+03 1/s. respectively under optimum conditions. The raw material cost for the CDA production The CDA production cost estimate through this process revealed purification step as the major cost intensive step in the entire process.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.03.144.

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