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Research Article

# Optimization of Monochloroacetic Acid Biodegradation by Recombinant *E. coli* BL21 (DE3)/pET-*bcf1* Carrying Haloacid Dehalogenase Gene from *Bacillus cereus* IndB1

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**ABSTRACT.** In recent years, attention to microbial dehalogenase has continually increased due to its potential application, both in bioremediation and in the biosynthesis of fine chemicals. Many microbial recombinant strains carrying dehalogenase gene have been developed, particularly to increase the dehalogenase production and its quality. In this study, we aimed to find the optimum condition for the production of active haloacid dehalogenase by *E. coli* BL21 (DE3) harboring recombinant plasmid pET-*bcf1* that carried haloacid dehalogenase gene from *Bacillus cereus* IndB1 local strain. This would be examined by assessing the ability of whole cell life culture to degrade monochloroacetic acid (MCA) and quantifying the chloride ion released into the medium. Several variables were evaluated to find this optimal condition. We found that the best condition for MCA biodegradation using this recombinant clone was at 0.2 mM MCA, 10  $\mu$ M of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), 6 hours of pre-induction incubation at 37°C with shaking, 2 hours IPTG induction at 30°C with shaking, at pH 7 in Luria Bertani (LB) liquid medium without NaCl, which produced about 0.056 mM chloride ions. Inducer concentration, pre-induction incubation time and temperature, as well as induction time and temperature were apparent to be associated with the expression of the protein, while the MCA concentration and the pH of the medium influenced the ability of the recombinant *E. coli* BL21 (DE3)/pET-*bcf1* to grow in toxic environment. Our findings laid the foundation for exploration of dehalogenases from local *Bacillus* strains through genetic engineering for MCA biodegradation.

**Keywords:** biodegradation, haloacid dehalogenase, monochloroacetic acid.

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## 1. Introduction

Monochloroacetic acid (MCA) is a derivative of carboxylic acid, produced by acetic acid chlorination. MCA is an industrially important organic compound, widely used as building blocks in the production of carboxymethyl cellulose (CMC), 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) herbicides, for paint and graffiti removal, plastic synthesis, and other forms as its ester or amide derivatives (European Commission, 2005). Nevertheless, these compounds are known to cause environmental pollution and harm to human health due to its toxicity, corrosive, persistence, and carcinogenic properties. These compounds along with other halogenated organic compounds, including MCA, are commonly found as pollutants in drinking water and wastewater (Islam *et al.*, 2020). High concentrations of MCA cause hazard to living organisms including humans (McRae *et al.*, 2004).

The presence of halogen in carboxylic acids increases its stability as well as its solubility in water. Naturally,

carbon-halogen bond could be cleaved through some chemical processes, such as oxygenation, oxidation, reduction, hydrolysis, and conjugation, which are catalyzed by enzymes (Guengerich, 1984). Dehalogenase is one group of enzyme that catalyzes the cleavage of carbon-halogen bonds in halogenated organic compounds (Slater *et al.*, 1997; Wang *et al.*, 2018). Some microorganisms have been found to be involved in this halogen removing process from haloalkanoic acid compounds in the environment by producing dehalogenase (Ismail *et al.*, 2017). The bioremediation of halogenated compounds by microorganisms was considered as an environmentally friendly approach, particularly in comparison to chemical processes (Abatenh *et al.*, 2017; Zhang *et al.*, 2014).

Attention to these microbial dehalogenases has increased continually due to their potential application, not only in bioremediation but also in biosynthesis of industrial fine chemicals (Adamu *et al.*, 2020; Ang *et al.*, 2018; Novak and Littlechild, 2013; Novak *et al.*, 2013). Furthermore, dehalogenases have also been developed for

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biosensor application, which is considered as a promising tool for halocarbon detection (Gul *et al.*, 2021). To increase the dehalogenase production and quality, the microbial recombinant strains harboring gene encoding dehalogenase have been developed (Ang *et al.*, 2018), particularly by cloning the target gene into a strong expression vector.

*E. coli* has been considered as the most suitable host for expressing stably folded globular proteins from various organisms (Rosano & Ceccarelli, 2014). Among available *E. coli* hosts, *E. coli* BL21 (DE3) is a superior host for recombinant protein expression (Shiloach *et al.*, 1996), which could produce the highest amount of specific recombinant proteins (Marisch *et al.*, 2013) with a simple set-up and low protease production. Expression of recombinant protein in this host relies on T7 RNA polymerase under the control of *lacUV5* promoter, which prevents any gene expression until induction is performed by adding IPTG into the cultures (Rosano & Ceccarelli, 2014). However, producing a large amount of soluble and functional recombinant proteins in *E. coli* host still presents challenges. Upon overexpression, the produced protein generally accumulates as insoluble aggregates known as the inclusion bodies in the bacterial cells, which is an inactive form of the enzymes. Therefore, to obtain a soluble active protein, finding an optimal condition that mimics its bacterial life's native condition is very important. Several experimental approaches have been developed to prevent the formation of aggregates, including reducing the inducer concentration (Winograd *et al.*, 1993), increasing the intracellular concentration of chaperon molecules (Mogk *et al.*, 2010), performing induction in mid-exponential phase or final exponential phase (Galloway *et al.*, 2003), lowering the induction temperature (Schein & Noteborn, 1988), and engineered the host cell (Rosano *et al.*, 2019).

In this study, we aimed to find an optimal condition required by the recombinant *E. coli* BL21 (DE3)/pET-*bcd1* to catalyze MCA biodegradation. The optimal condition was studied by varying inducer and substrate concentrations, pre-induction incubation time and temperature, induction time and temperature, and pH of the utilized medium. Degree of MCA biodegradation was examined by determining the chloride ions released into the medium using a UV-Vis spectrophotometer according to Bergmann and Sanik method (Bergmann & Sanik, 1957). We expected that the optimal condition would promote the production of dissolved and active haloacid dehalogenase, hence the clone would be performing its best in degrading MCA.

## 2. Materials and Methods

### 2.1 Source of the recombinant microorganism

The recombinant clone of *E. coli* BL21 (DE3) carrying the haloacid dehalogenase gene from *Bacillus cereus* IndB1 local strain in pET-30(a) expression vector, termed as *E. coli* BL21 (DE3)/pET-*bcd1*, was obtained from our previous research (Ratnaningsih & Idris, 2018). The *bcd1* haloacid dehalogenase gene in this recombinant clone has been sequenced and the sequence has already been deposited in GenBank (accession number KU49803). Initially, this gene was cloned into pGEM-T vector in *E.*

*coli* TOP10 host for analysis, which then subsequently sub-cloned into pET-30a(+) expression vector in *E. coli* BL21 (DE3) for expression with T7 RNA polymerase under the control of *lacUV5* promoter. The clone has been stored at  $-80^{\circ}\text{C}$  in 20 % glycerol-LB medium (v/v) as a stock culture.

### 2.2 Regenerating the *E. coli* BL21 (DE3)/pET-*bcd1* culture

The glycerol stock culture of the recombinant *E. coli* BL21 (DE3)/pET-*bcd1* was inoculated into 2 mL of LB liquid medium (10 g/L tryptone and 5 g/L yeast extract, without NaCl) containing 50  $\mu\text{g}/\text{mL}$  kanamycin. The culture was incubated overnight at  $37^{\circ}\text{C}$  with shaking. A single colony for further experiments was obtained by streaking this fresh culture onto a solid LB medium containing 50  $\mu\text{g}/\text{mL}$  kanamycin and followed by overnight incubation at  $37^{\circ}\text{C}$ . PCR analysis employing the primers used for initial gene isolation (which are 5'-ATGGATGGAACACTACTATC-3' as forward primer and 5'-TTATTTACTAGATGAAGTTTG-3' as reverse primer) as well as restriction analysis using *EcoRI* and *HindIII* were accordingly performed for clone confirmation.

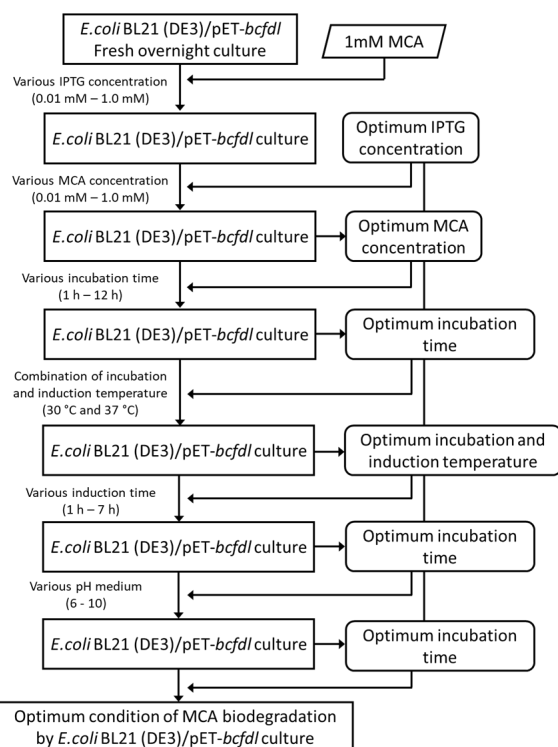
### 2.3 Growth of *E. coli* BL21 (DE3)/pET-*bcd1* in MCA containing medium

Growth of recombinant *E. coli* BL21 (DE3)/pET-*bcd1* in the medium containing various MCA concentrations was studied by culturing the clone in 2 mL LB broth (without NaCl) containing 50  $\mu\text{g}/\text{mL}$  kanamycin. The MCA concentration was varied from 1 mM – 10 mM. Inoculation was performed using 10  $\mu\text{L}$  of *E. coli* BL21 (DE3)/pET-*bcd1* overnight fresh culture, followed by 6 hours incubation at  $37^{\circ}\text{C}$  with shaking. Ability of the clone to grow in MCA containing medium was observed by measuring culture optical density at 600 nm, called as OD<sub>600</sub>. The absence of MCA in the medium was used as the growth control.

### 2.4 MCA biodegradation by *E. coli* BL21 (DE3)/pET-*bcd1*

MCA biodegradation was studied utilizing *E. coli* BL21 (DE3)/pET-*bcd1* whole cell culture. Biodegradation experiments were carried out in 2 mL LB liquid medium (without NaCl) containing 50  $\mu\text{g}/\text{mL}$  kanamycin, seeded with 10  $\mu\text{L}$  overnight fresh culture of *E. coli* BL21 (DE3)/pET-*bcd1*. Series of variables and steps of optimization is depicted in Fig. 1.

Our standard condition in performing optimization for MCA biodegradation was as follows. All experiments were carried out in 0.2 mM of MCA, except for IPTG optimization, which was performed with 1 mM MCA. Prior to induction, the culture was incubated at  $37^{\circ}\text{C}$  for 6 hours with shaking. At this step, 1 mL of this culture was drawn into a new sterile tube and stored at  $4^{\circ}\text{C}$  to be used as a control. Then, the IPTG inducer was added to the culture at a 10  $\mu\text{M}$  final concentration and the incubation was continued at  $30^{\circ}\text{C}$  for 2 hours. The culture was then placed on ice for 10 minutes, centrifuged, and the supernatant was collected.



**Fig 1.** Steps of optimization on MCA biodegradation by *E. coli* BL21 (DE3)/pET-*bcfd1*

This centrifugation and supernatant collection was also performed identically using the control. Then, 1 mL of the supernatant was transferred into a new tube and the amount of chloride ion released into the medium was quantified. The optimum condition of MCA biodegradation was determined by varying one particular variable, while others were set at the standard conditions. As shown in Fig.1, the varying variables were inducer (IPTG) concentration, substrate (MCA) concentration, pre-induction incubation period and temperature, induction period and temperature, and pH of the medium. All experiments were conducted in two replicates.

### 2.5 Chloride ion determination

Chloride ion determination was performed according to the method introduced by Bergmann and Sanik (Bergmann & Sanik, 1957) as follows. Into 1 mL of the culture supernatant, 0.1 mL of 0.1% (w/v)  $\text{Hg}(\text{SCN})_2$  was added, mixed well by inverting the tube several times, and incubated for 5 minutes at room temperature. Then, 0.1 mL of 0.25 M  $\text{FeNH}_4(\text{SO}_4)_2$  in 9 M  $\text{HNO}_3$  was added into the mixture, mixed well by inverting the tube, and further incubated for 5 minutes at room temperature. Absorbance of the resulted coloured solution was measured at 460 nm, and the chloride ion concentration was determined using NaCl standard curve.

## 3. Results and Discussions

### 3.1 Growth of *E. coli* BL21 (DE3)/pET-*bcfd1* in MCA containing medium

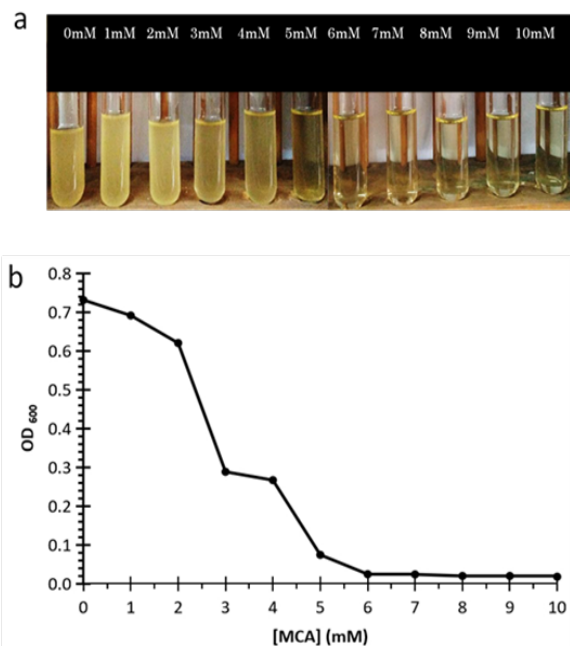
The growth of *E. coli* BL21 (DE3)/pET-*bcfd1* in LB liquid medium (without NaCl) containing various MCA

concentrations was evaluated prior to optimization of MCA biodegradation. This assay was conducted in order to observe the highest MCA concentration that can be tolerated by the recombinant *E. coli* BL21 (DE3)/pET-*bcfd1*. The result of growth is shown in Fig. 2(a) and the cells' turbidity of the culture was measured by determining culture's optical density at 600 nm ( $\text{OD}_{600}$ ) as shown in Fig. 2(b). It could be seen in Fig. 2(a) that culture's turbidity tends to decrease with incremental increase of MCA concentration, indicating that MCA was toxic to the cells. This observation was supported by culture's  $\text{OD}_{600}$ , as shown in Fig. 2(b). At 1mM and 2 mM of MCA, the recombinant *E. coli* BL21 (DE3)/pET-*bcfd1* was still apparent to grow quite well, but it already lost half of its growth upon increasing the MCA concentration to 3 mM. There was no growth observed when the MCA concentration was  $\geq 6$  mM. Other previous research has reported that *Bacillus* sp. TW1 was able to grow in a medium containing 0.5 mM of MCA (Zulkifly *et al.*, 2010), which was lower compared to *Arthrobacter* sp. D2 that was able to tolerate up to 20 mM of MCA (Alomar *et al.*, 2014). These reports indicated that tolerable MCA concentration could be different from strain to strain. It was also reported that MCA dehalogenase activity was found to be inducible, either in wild type of *Pseudomonas* sp. R1 (Ismail *et al.*, 2008) or in its recombinant clone of *Burkholderia* species MBA4 (Su *et al.*, 2013). Based on our result, we chose 1 mM of MCA concentration for initial optimization of inducer (IPTG) concentration.

### 3.2 Optimization of inducer concentration

Inducer concentration was optimized by performing experiments at various concentrations of inducer (IPTG, in the range of 0 mM to 1 mM). The result is presented in Fig. 3(a). Biodegradation was represented as chloride ion concentration released into the medium. It was apparent that the amount of IPTG influenced MCA biodegradation. Interestingly, a higher MCA biodegradation occurred at a lower inducer concentration, hence a high inducer concentration did not appear to promote MCA overexpression. This finding was attributed to the overexpression of recombinant protein in *E. coli* BL21 (DE3)/pET-*bcfd1* that was performed by T7 RNA polymerase under the control of *lacUV5* promoter (Ma *et al.*, 2020), which was regulated by the inducer (IPTG). Though protein expression might have been increased at higher inducer concentration, overexpression of the protein would instead lead to inclusion bodies formation, which rendered the enzyme inactive (Ma *et al.*, 2020; Rosano *et al.*, 2014).

It is well known that the slow rate of protein synthesis in intracellular expression system might reduce the formation of insoluble nonfunctional recombinant proteins (inclusion bodies). Hence, reducing the speed of protein synthesis was the main strategy to obtain functional recombinant proteins (Rosano & Ceccarelli, 2014). Therefore, the lowest inducer concentration is much better to express such functional recombinant proteins (Riggs *et al.*, 1994). This fact is in good agreement with our findings. We obtained that the highest MCA biodegradation performed by *E. coli* BL21 (DE3)/pET-*bcfd1* was achieved at 10  $\mu\text{M}$  of IPTG, which was used for further analysis.



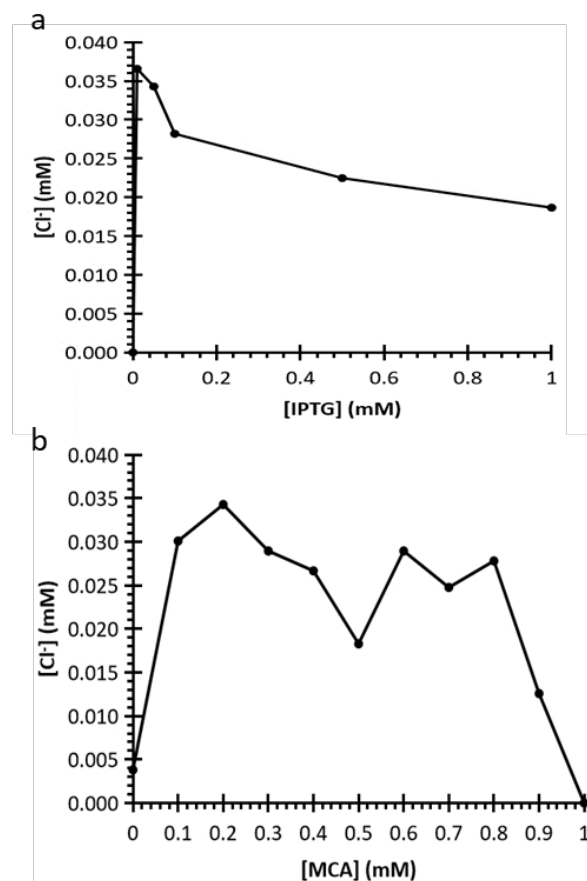
**Fig. 2** The growth of the recombinant *E. coli* BL21 (DE3)/pET-*bcd1* in LB medium containing various concentration of MCA observed after overnight incubation at 37°C with agitation. (a) The turbidity of the culture and (b) the OD<sub>600</sub> of the culture.

### 3.3 Optimization of MCA concentration

Optimal MCA concentration for biodegradation was investigated in LB liquid medium (without NaCl) induced by 10 μM IPTG. The MCA concentrations were varied from 0 mM to 1 mM, and other variables were kept constant as stated in materials and methods. The result is presented in Fig. 3(b). The optimum MCA concentration for best biodegradation by *E. coli* BL21 (DE3)/pET-*bcd1* was found to be 0.2 mM, as indicated by the highest concentration of chloride ion released into the medium. The MCA biodegradation seemed to decrease with the increase of MCA concentration. Although the growth of *E. coli* BL21 (DE3)/pET-*bcd1* was still observed at 1 mM of MCA, no MCA biodegradation occurred under this condition, indicating that 1 mM of MCA was already toxic to the cells. Though the bacteria could take up MCA as additional carbon source, a high MCA concentration could become toxic to the cells (Torz & Beschkov, 2005).

### 3.4 Optimization of pre-induction incubation time

Under the optimal concentration of inducer and substrate, which were 10 μM of IPTG and 0.2 mM of MCA respectively, MCA biodegradation was investigated by varying the pre-induction incubation time while keeping all other variables constant (as stated in materials and methods). The result is presented in Fig. 4(a), which clearly indicated that pre-induction incubation period influenced MCA biodegradation. This incubation period was associated with bacterial growth phase prior to induction for expressing the haloacid dehalogenase gene. Our result indicated that the best pre-induction incubation time was 6 hours at 37°C. Prolonged pre-induction incubation time at this condition did not appear to increase the MCA biodegradation.



**Fig. 3** MCA biodegradation by *E. coli* BL21 (DE3)/pET-*bcd1* in various concentrations of (a) IPTG inducer and (b) MCA substrate.

The number of cells and stages of cell growth would affect the level of inducible gene expression. Cells in the middle exponential phase are ideal for expression because in this stage the cells are actively dividing. It was known that enzyme production will increase by increasing the cell concentration until it reached the stationary phase, and addition of inducer at the final exponential phase (close to the stationary phase) did not have any significant effect on enzyme production (Yildir *et al.*, 1998). In addition, when the synthesis of recombinant protein and cell growth simultaneously occurred, which happened if the induction was carried out at early incubation time, protein synthesis and cell growth would tend to be slow because growth resources have to be divided to support both processes (Fazaeli *et al.*, 2019). In our experiments, this fact could be seen at the 1<sup>st</sup> to 4<sup>th</sup> hour of incubation, where the amounts of the released chloride ions were low. In agreement with a previous report, our experiment showed that after 6 hours of incubation, the culture OD<sub>600</sub> has reached 0.6 – 0.8, which was optimum for the induction (Galloway *et al.*, 2003).

### 3.5 Optimization of pre-induction and induction temperature

To study the effect of incubation temperature prior to induction and after IPTG induction on MCA biodegradation, experiments were performed by varying these two variables at a standard biodegradation

condition as stated in the materials and methods. Duration of pre-induction and after induction incubation was kept to 6 hours and 2 hours respectively. The result is presented in Fig. 4(b). The best MCA biodegradation was observed at 37 °C pre-induction incubation followed by shifting to 30 °C incubation after induction. Incubation temperature prior to IPTG induction was clearly associated to bacterial growth rate, whereas condition after the addition of IPTG was related to the rate of recombinant protein expression. This result was consistent with previous report on the optimal temperature growth for *E. coli* cells, which is 37°C (Noor *et al.*, 2013). Conversely, lower incubation temperature after the induction would reduce the rate of protein synthesis, thus reducing the accumulation of recombinant protein and increasing protein solubility (Pacheco *et al.*, 2012; Sahdev *et al.*, 2008). This condition might promote correct protein folding and therefore increase MCA biodegradation.

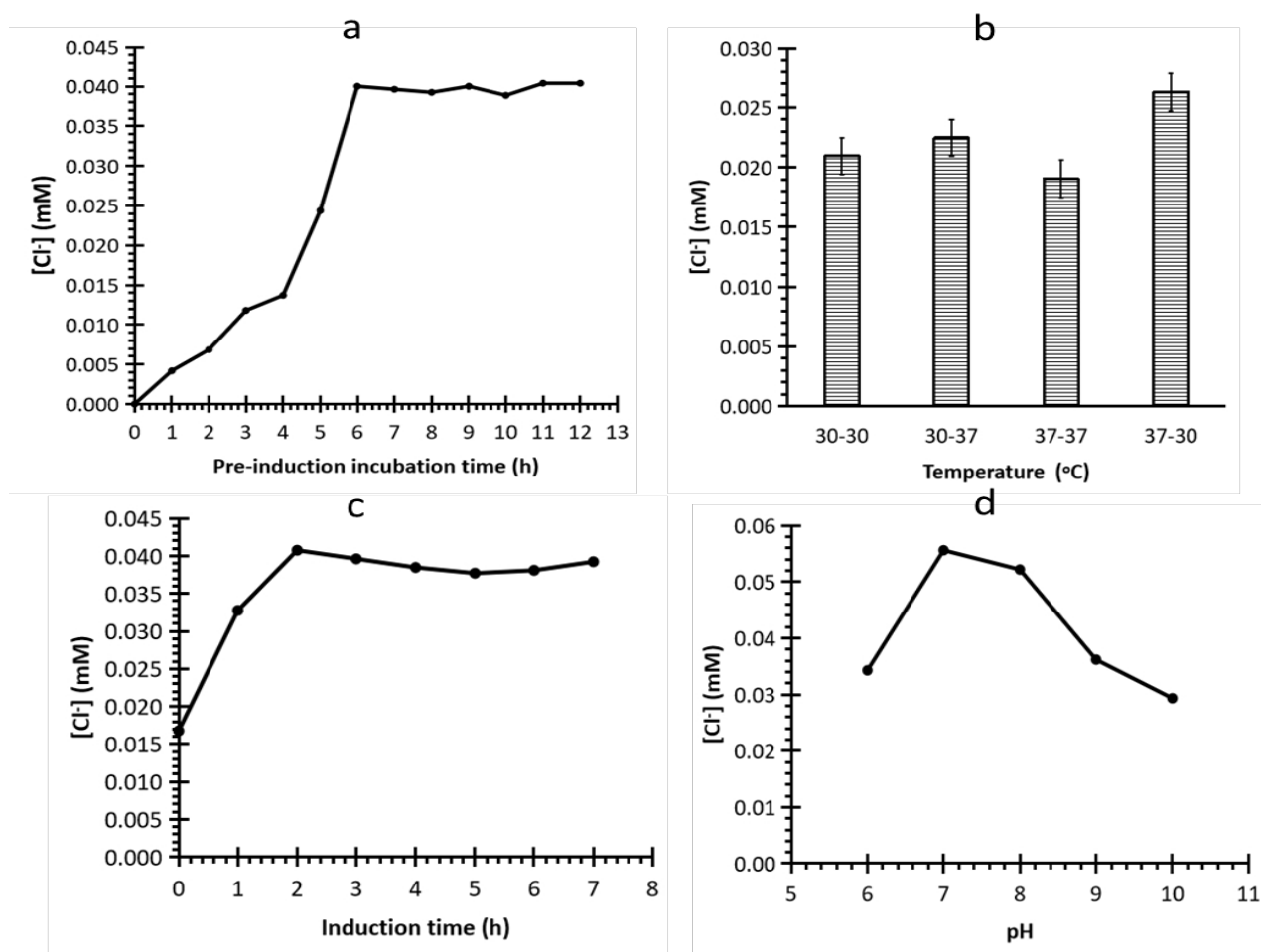
### 3.6 Optimization of induction incubation time

Induction incubation time was defined as the incubation period after IPTG addition. The induction incubation was varied from 1 to 7 hours by keeping all other variables constant. The result is presented in

Fig.4(c), which clearly indicated that the best induction incubation time for MCA biodegradation by *E. coli* BL21 (DE3)/pET-*bcd1* is 2 hours. Prolonged incubation after the induction did not increase MCA biodegradation. Extending induction period might have triggered the formation of inclusion bodies as the recombinant proteins accumulated in the cells. Reducing the duration of the induction time is a strategy to obtain recombinant protein in its active state (Gutiérrez-González *et al.*, 2019).

### 3.7 Optimization of medium pH

The effect of pH in MCA biodegradation was studied at a standard condition (as stated in materials and methods) by varying the medium pH from 6 to 10. The result is presented in Fig. 4(d). It could be seen that highest MCA biodegradation occurred at pH 7 which released ~0.056 mM chloride ions to the medium. Apparently, the *E. coli* BL21 (DE3)/pET-*bcd1* drastically lost its ability to degrade MCA when the pH of the medium was acidic (pH 6) or alkaline (pH 9). We attributed this finding to the growth difficulty of *E. coli* BL21 (DE3) in acidic or alkaline environment. It was known that the most common pH condition that supports the growth of *E. coli* BL21 (DE3) was in the range of 6.5 to 7.5 (Wang *et al.*, 2014).



**Fig. 4** Biodegradation of MCA by *E. coli* BL21 (DE3)/pET-*bcd1* at (a) different pre-induction incubation time, (b) combination of temperature on pre-induction and after induction incubation, (c) induction incubation time, and (d) pH of the growth medium.

## 6. Conclusion

The whole-cell culture of the recombinant *E. coli* BL21 (DE3)/pET-*bcd1* carrying the gene from *Bacillus cereus* IndB1 local strain was able to degrade MCA. Our studies found that the best condition for MCA biodegradation using this recombinant clone was at 0.2 mM MCA, 10  $\mu$ M of IPTG, 6 hours of pre-induction incubation at 37°C with shaking, followed by 2 hours IPTG induction at 30°C with shaking, at pH 7 in LB liquid medium without NaCl, which produced ~0.056 mM chloride ions. The reported findings provide useful foundation for recombinant enzyme production to facilitate further studies on engineered haloacid dehalogenase for MCA biodegradation. In particular, our result provides optimum condition to utilize whole cell culture of recombinant *E. coli* BL21 (DE3)/pET-*bcd1* for MCA biodegradation. Enzyme availability and enzyme activity within the cells still require further studies. The production of haloacid dehalogenase in this recombinant clone might be further increased by cloning the gene into a stronger expression vector or utilizing other *E. coli* host with more favourable characteristics.

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