Original Article

Expression Optimizing of Recombinant Oxalyl-CoA Decarboxylase in *Escherichia coli*

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Abstract

Background: One of the most common diseases of the urinary tract is stones of this system, including kidney stones. About 70%–80% of kidney stones are calcium oxalate. Oxalyl-CoA decarboxylase is a single polypeptide included of 568 amino acids which play a key role in oxalate degradation.

Materials and Methods: The aim of current study is high-level expression of oxalyl-CoA decarboxylase in *Escherichia coli* BL21 (DE3). To achieve this aim, oxalyl-CoA decarboxylase gene was cloned upon pET-30a (+) with T7 promoter. The vector containing the oxalyl-CoA decarboxylase gene was transformed into *E. coli* and the expression of the gene was examined on a laboratory scale and fermentor. At first, the effect of temperature, culture medium, and induction time on oxalyl-CoA decarboxylase expression at three levels was examined.

Results: The obtained data showed that the highest expression was related to the terrific broth culture medium and temperature of 32°C with an inducer concentration of 1 mM. Under this situation the ultimate cells dry weight and the final oxalyl-CoA decarboxylase expression were 2.46 g/l and 36% of total protein, respectively. Then induction time was optimized in a bench bioreactor and productivity of oxalyl-CoA decarboxylase was calculated. Under optimized condition the cell density, biomass productivity and oxalyl-CoA decarboxylase concentration reached 4.02 g/l, 0.22 g/l/h, and 0.7 g/l which are one of the highest reported rates.

Conclusion: This study demonstrated that high levels of oxalyl-CoA decarboxylase can be achieved by optimizing the expression conditions.

Keywords: *Escherichia coli*, overexpression, oxalate, *Oxalobacter formigenes*, oxalyl-CoA decarboxylase

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Introduction

One of the most common diseases in the field of urology is kidney stones, which are caused by a large number of people in developed or developing societies every year.[1,2] Kidney stones are found in both men and women but are more likely to occur in men or with age.[3] Kidney stones can be calcium oxalate, calcium phosphate, cysteine, or uric acid.[4] The type of stone plays a main role hampering recurrence of the stone. Studies have shown that about 75% of kidney stones are calcium oxalate.[5] *Oxalobacter formigenes* is a gram-negative and anerobic bacterium that has a key function in the breakdown of oxalate, resulting in the re-emergence of stones, due to the presence of the two enzymes oxalyl-CoA decarboxylase (OXC) and formyl-CoA decarboxylase (FRC).[6] Previous studies have exposed that people who are recurrent for oxalate kidney stones have lower levels of oxalyl-CoA decarboxylase enzyme.[7] Oxalyl-CoA decarboxylase is a four-unit enzyme that requires the thiamine pyrophosphate cofactor, which has a molecular weight of 60 kDa.[8-10] This enzyme can be used in diagnostic kits to evaluate the level of the oxalyl-CoA decarboxylase enzyme in people who commonly suffering oxalate stones.[11] Foster *et al.*

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demonstrated that Arabidopsis oxalyl-CoA Decarboxylase is crucial for Oxalate Catabolism in Plants.[12] The ultimate goal of this project is to use oxalyl-CoA decarboxylase enzyme in diagnostic kits, the first step is to achieve a high level of enzyme expression. For this aim, we optimized the expression level of the oxalyl-CoA decarboxylase enzyme using the recombinant strain of *Escherichia coli* containing this enzyme and investigated the enzyme expression level.

Since various factors such as culture medium, temperature, induction time, and inductor concentration are effective in the expression of recombinant protein,[13,14] the effect of these factors in the expression of OXC enzyme was investigated in both flask and fermentor. The outcome of this study demonstrated that by optimizing the expression conditions, a high level of the OXC enzyme can be achieved.

Materials and Methods

Required chemicals were prepared from Merck and restriction enzymes from fermentase. *E. coli* BL21 (DE3) was used as host.

Recombinant protein production

For cloning, first, the sequence of oxalyl-CoA decarboxylase gene related to *Oxalobacter formigenes* was retrieved from National Center for Biotechnology Information. This sequence was cloned in the pET30a (+) vector between the *NdeI* and *EcoRI* restriction enzyme site. In designing the gene cassette, an octet sequence of histidine was considered for ease of purification. The resulting recombinant vector was transformed into *E. coil* BL21 (DE3) by calcium chloride method. One of the single transformed clones was considered for expression and an overnight culture was prepared. 200 µl of overnight culture was used as seed for 10 ml fresh culture and when it reached the desired density (OD_{600} = 0.7), protein expression was induced by 0.1 mMIPTG. 2 h after induction, 1.5 ml of the culture medium was taken and centrifuged (6000 g, 10 min, and 4°C). A sample buffer (1 (w/v) % sodium dodecyl sulfate (SDS), 0.5 (w/v) % Bromophenol blue, 10 (v/v) % glycerol, 0.25 M Tris-HCl pH 6.8, 5 (v/v) % B-mercaptoethanol) were added to the precipitate and protein expression was examined on 12% (SDS- polyacrylamide gel electrophoresis [PAGE]).[15]

Expression optimization in small-scale

Expression optimization was first performed on a flask scale. For this purpose, overnight culture was prepared first. The overnight culture was subculture and after reaching the desired density of OD_{600} 0.7, induction was performed in 50 ml of Luria-Bertani medium (LB), terrific broth (TB) and 32Y C media at 28° C, 37°C and 32°C with inductor concentrations of 0.1, 0.5 and 1 mM. The basis for these selections (temperature, culture medium, and inductor concentration) was similar articles. To calculate the dry weight, 10 cc of the culture medium was centrifuged at 6000 rpm for 10 min. The resulting precipitate was separated and transferred to a weighted aluminum foil. The precipitate was allowed to dry completely. After complete drying, the aluminum sheet was weighed again.

Optimization of oxalyl-CoA decarboxylase expression in fermentor

Inoculation fluid was first prepared for culture in the fermentor (Infors). For this purpose, a single colony was first cultured overnight in TB medium containing 50 µg/mL of kanamycin. The overnight culture was subculture in 200 ml of TB medium and added to the fermentor when it density reached to OD600 nm = 0.7. The fermentor contained 1 liter of TB medium and 50 µg/mL of kanamycin. The pH of the medium was kept at7 ± 0.05 (using HCl 1N or NaOH 1M). Silicone oil (0.1% [v/v]) was used to prevent foaming. The stirrer speed was set to 800–900 rpm and dissolved oxygen was run at 30%–40% of air saturation. When cell density of culture reached 0.16, 1.05, and 1.38 g/L, the temperature was decreased to 32°C and0.5 mMIPTG added for protein expression. The cells were collected through centrifugation at 6000 g for 10 min at 4°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis

Electrophoresis was run in 1 mm gels at 100V. Gel stained by coomassie (G250) staining. Bradford assay was used for calculating total protein. For western blotting, proteins were first transferred to nitrocellulose paper using transfer buffer (20 mM Tris, 15 mM Glycin, 20 (v/v) % Methanol, pH = 8) and then the paper was blocked with 3 (w/v) % bovine serum albumin and 0.05 (v/v) % Tween in tris-buffered saline. Primary antibodies (Mouse anti-His-tag antibody) were added and after washing, secondary antibodies (goat anti-mouse horseradish peroxidase) were added.[16]

Purification of recombinant OXC

Histidine tag was used in the designed gene cassette to facilitate the purification of recombinant OXC. Histidine tends to be nickel, so this property was used for purification. Denaturing buffer (2 M Urea, 50 mM NaH2PO4, 300 mMNaCl, pH 7.9) was added to the cell precipitate from 10 ml of culture medium and allowed to mix gently on a shaker at 4°C. The solution was then sonicated on ice (20s pulses 30s), and the supernatant from the sonicated (12,000 rpm for 40 min) mixture was poured into a chromatographic column. After passing the sample, the column was washed and eluted with a high concentration of imidazole (250 mM imidazole, 50 mM NaH2PO4, 300 mMNaCl, pH 7.9). Fraction of eluted proteins were analyzed by SDS–PAGE and dot blot.

Results

Designing and cloning of recombinant OXC

After obtaining the OXC gene sequence of *O. formigenes* (Gen Bank: M77128.1), this sequence was ordered and cloned in pET30a (+) vector. *NdeI* and *EcoRI* Enzymes were selected for cloning, Vector NTI 11.0 software was used for designing cloning. Using this software, the cleavage enzymes were examined that have no site on the gene sequence. Codon bias was observed for the *E. coli* host, the amount of GC and the

efficiency of translation conditions (absence of the initial methionine codon in the stem structure) were investigated. Figure 1 describes the schematic diagram of the OXC gene which was cloned in pET30a.

Recombinant OXC expression

To examine OXC expression, the vector was transformed into *E. coil* BL21 (DE3). SDS-PAGE was used to evaluate expression [Figure 2] and protein expression was confirmed by Western blot [Figure 3].

Purification of OXC protein

The recombinant OXC protein has a histidine tag which was used for purification by using Ni-NTA chromatography. The eluted was analyzed by dot blot that confirms protein purification [Figure 4].

OXC expression optimizing

In general, in the expression of recombinant proteins, various factors such as temperature, inductor concentration, culture medium composition, and induction time can play a role. Therefore, in this study, OXC expression was first investigated at the flask scale at three temperatures, three culture media, and three inductor concentrations. Table 1 lists all the conditions of the tests performed and the final expression percentage, dry weight, and final density obtained.

One of the factors that can have a significant effect on protein expression is the point at which the growth curve is induced. In this study, the expression of OXC at three different points of the growth curve was investigated. These points were considered the beginning of the log phase, the middle of the log phase, and the end of the log phase. It should be noted that these induction points were obtained based on the *E. coli* BL21 (DE3) growth curve in TB medium at 32°C without the presence of an inductor. Figure 5 shows the effects of induction on biomass productivity and the final cell in batch cultures. Figure 6 shows the effects of induction time on cell growth and OXC expression. By induction at a cell density of 1.05 g/L, the most biomass productivity was obtained.

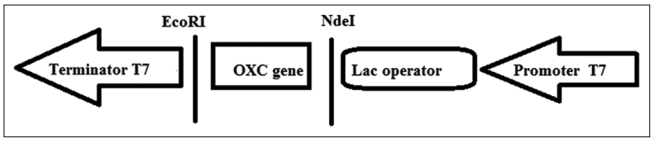

Figure 1: The schematic diagram of the OXC gene which was cloned in pET30a

Discussion

Recombinant proteins have led to a major revolution in the treatment of disease.[17,18] Kidney stones are one of the most important diseases of the urinary tract, which are largely due to nutrition, race, geographical conditions, and lifestyle.[19] In addition to much suffering, the disease imposes great economic costs on societies. Oxalate stones make up the largest percentage of kidney stones. Oxalate is broken down by two enzymes from the *O. formigenes*, two of which

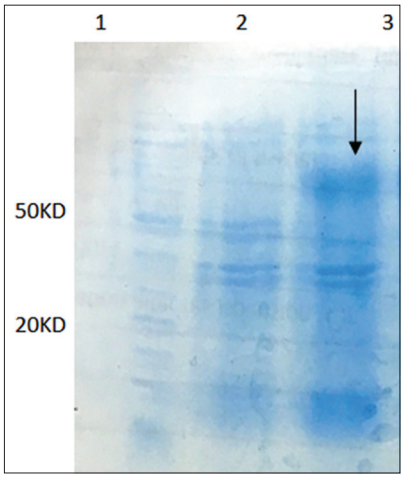

Figure 2: Analysis of OXC expression by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane 1; protein ladder; lane 2 previous to induction; lane 3 two hour after induction

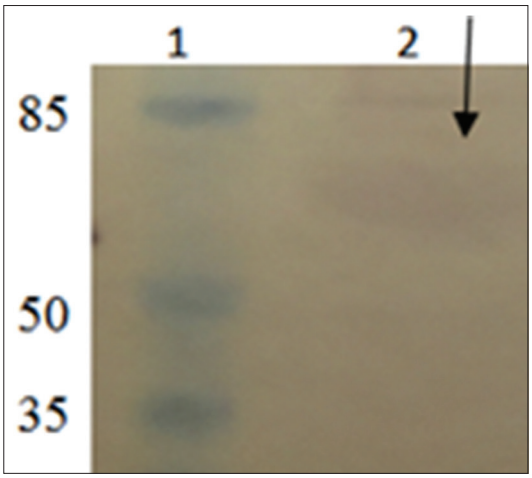

Figure 3: Analysis of OXC expression by Western blot. Lane 1; protein ladder; Lane 2; expression after 2h

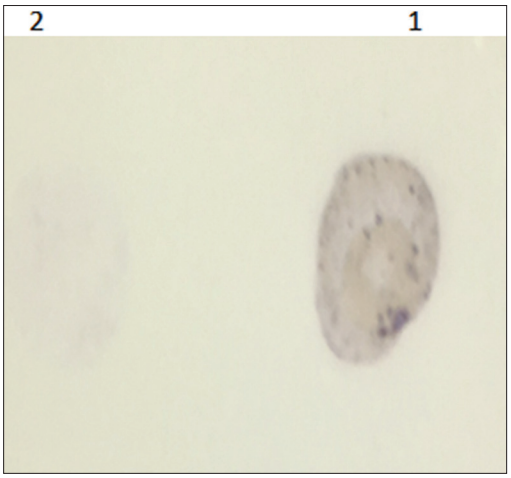

Figure 4: Dot blot analysis of OXC purification by chromatography. Lane 1; eluted OXC. Lane 2; negative control

Table 1: Effect of different condition on recombinant oxalylCoA decarboxylase expression

Expression number	Growth temperature (°C)	Medium	IPTG concentration (mm)	Final OD600	Final cell density (g DCW/L) DCW	Percent of OXC expression
1	28	LB	0.1	2.4±0.1	1.10±0.02	19
2	28	TB	0.5	3.9±0.1	1.79±0.04	20
3	28	32Y	1	2.5±0.1	1.09±0.03	19
4	32	LB	0.5	3.1±0.1	1.42±0.05	25
5	32	TB	1.0	5.35±0.1	2.46±0.04	36
6	32	32Y	0.1	3.7±0.1	1.70±0.02	26
7	37	LB	1.0	2.5±0.1	1.15±0.03	24
8	37	TB	0.1	4.3±0.1	1.97±0.06	27
9	37	32Y	0.5	2.9±0.1	1.33±0.03	25

DCW: Dry cell weight, OXC: OxalylCoA decarboxylase, LB: Luria-Bertani medium, TB: Terrific Broth, IPTG: Isopropyl β-D-1-thiogalactopyranoside

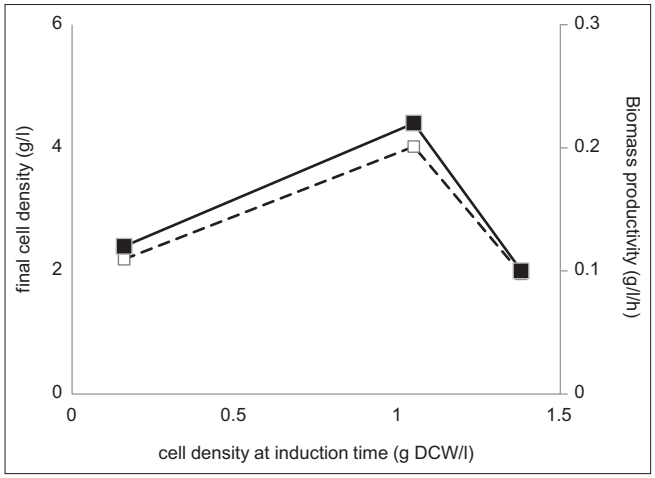

Figure 5: The effects of induction time (cell density at induction time g DCW/L) on the biomass productivity (g DCW /L/h) (black square) and the final cell density (g DCW /L) (white square) in batch cultures

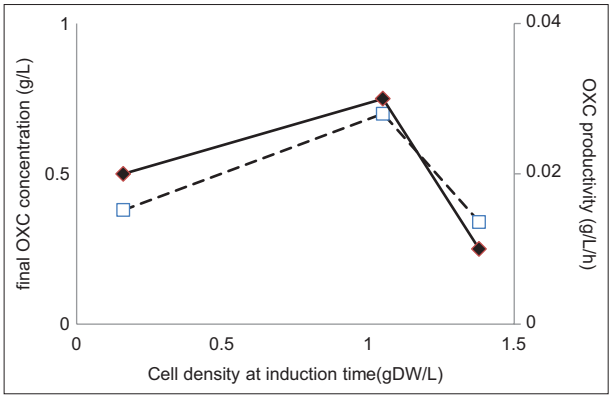

Figure 6: The effects of induction time (cell density at induction time g DCW/L) on the OXC productivity (g/L/h) (white square) and final OXC concentration (g/L) (black square) in batch cultures of Escherichia coli BL21 (DE3)

are OXC and FRC.[20] Previous studies have examined the presence or absence of the *O. formigenes* in people who have persistent kidney stones.[21] These studies are ineffective because they did not examine the presence or absence of OXC enzymes. Since there may be bacteria, the enzyme is disabled for any reason. In 2019 study by Abarghooi Kahaki *et al*. examined the presence of the OXC enzyme in people who constantly suffering from kidney stones. By using a designed enzyme-linked immunosorbent assay kit, they showed that the OXC enzyme levels in these people were significantly reduced.[11] The designed kit requires an OXC enzyme, and the current study looked at ways to increase the expression of this enzyme. Since *E. coli* is the most common and economical host of recombinant protein expression, this host was chosen to express recombinant OXC.[22,23] Various factors can affect the growth and expression of recombinant protein in *E. coli*, including temperature, culture medium, inducer concentration, and induction time. This study demonstrated that the best conditions for growth do not necessarily mean the best conditions for expression.[24] The best growth temperature for *E. coli* is 37°C, while the best temperature for enzyme expression is 32°C. Lower temperatures played an important role in increasing expression. Cultivation medium compounds also have a significant effect on protein expression. Among the selected culture medium, TB culture medium had the highest percentage of recombinant protein expression, i.e., 36%. The reason for the high expression in the TB culture medium is that it is rich in yeast extract, which can be used as a source of nitrogen to make recombinant proteins.[25] In addition, one of the restrictive factors for the growth of *E. coli* is the acidification of the environment. The TB culture medium, due to its phosphate buffer, prevents changes in the pH of the environment to some extent. As a result, the bacterium is allowed to grow and produce more recombinant protein.[25] The expression of the oxalate gene is controlled by the operonlacI, which is induced by the combination of IPTG. The inductor concentration also plays a main role in the expression effect. Under low concentrations, the promoter does not turn on, and the use of higher inductor concentrations is not economically justified. Since the TB culture medium and temperature of 32°C showed the highest percentage of expression in the flask, the same conditions were considered for cultivation in the fermentor and the induction time in the fermentor was optimized. Three times the induction time, which was the beginning (0.16 g/l), middle (1.05 g/l), and end (1.35 g/l) of the log phase, were considered. The highest expression efficiency

was related to the time of the middle phase. Induction prevents growth at the beginning of the log phase, and induction at the end of the log phase decreases due to the entry of cells into the stationary phase of expression. Cell density in the induced state in the middle of the log phase (1.05 g/l) is around 2 times the induction at the beginning (0.16 g/l) or end (1.35 g/l) of the log phase. Under these conditions, the cell density, biomass productivity and OXC concentration reached 4.02 g/l, 0.22 g/l/h, and 0.7 g/l which are one of the highest reported rates.

Conclusion

The OXC enzyme can be considered as a marker enzyme in people who regularly develop kidney stones. The plan of this study was to supply a solution for the high expression of this enzyme so that a sufficient amount of it is available for use in kits. This study demonstrated that high levels of this enzyme can be achieved by optimizing the expression conditions.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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