Development of a Novel and Efficient HCDC Process for Heterologous Production of Ambystoma Mexicanum Epidermal Lipoxygenase (AmbLOXe) in *E. coli*

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ABSTRACT: *LOXe enzyme is responsible for the regrowth of severed limbs and is a new candidate in wound healing. This study developed an efficient feeding approach in the fed-batch culture of E. coli with the maximum achievable specific growth rate to obtain higher production and productivity. In this method, the specific growth rate before the induction of the TB culture medium was maintained at a maximum of 0.92 ± 0.2 h^{-1}. Then, with the simultaneous initiation of induction and feeding, due to system limitations, the specific growth rate decreased over time until it reached less than 0.1 until growth finally stopped. The complex medium containing glucose 200 g/l and yeast extract 200 g/L was used as the feeding medium. Using this strategy, the total production and productivity of LOXe increased from 5.4 to 27.35 g/L and from 0.527 to 2.19 g/L, respectively, compared to batch culture. By investigating the effect of glucose and yeast extract concentrations in the feeding medium, despite the 50% decrease in yeast extract, recombinant enzyme production increased by about 10% from 27.35 to 30.43 g/L. Finally, optimization of the inducer amounts increased rLOXe production (20% increase) to 36.4 g/L and overall efficiency to 2.71 (11.5% increment). This is one of the highest productivities ever reported for the recombinant protein production of E. coli.*

KEYWORDS: *Amphibian epidermal lipoxygenase (AmbLOXe), Overall productivity, Recombinant E. coli, Fed-batch, Feeding strategy.*

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1021-9986/2023/12/4354-4366 13/$/6.03

INTRODUCTION

Lipoxygenases (EC 1.13.11.12; LOXs) are a family of non-heme, non-sulfur monomeric iron dioxygenases that catalyze the conversion of polyunsaturated fatty acids. Lipoxygenases are widely found in plants, fungi, and animals [1,2]. Inhibition of LOX in skin cells reduces cell migration and proliferation, so this group of enzymes appears to play a key role in wound healing [1,3]. In one study, the effects of the lipoxygenase enzyme in the highly related amphibian Mexican salamander (Ambystoma mexicanum) for tissue repair and wound healing in humans have been demonstrated in vitro [4-6]. Adult salamanders have a high ability to regenerate all body parts, tail, central nervous system, eyes, and heart tissue [1,2,5]. These animals significantly heal wounds without leaving scars on their bodies. In vitro and in vivo studies have shown that Ambloxe Lipoxygenase Amphibian Epidermal improves wound healing in mammals [1,5].

Fed-batch culture is the preferred method to achieve high cell density and recombinant protein over-production. Despite the numerous benefits of the fed-batch fermentation process due to the difficulty of precisely controlling the concentration of raw materials and oxygen and consequently limiting or increasing the need for raw materials (especially carbon sources) for bacterial growth, CO_2 production, and high heat, high demand and limited oxygen transfer capacity, extreme viscosity, reduced mixing efficiency, plasmid instability and its increase with increasing production duration, product degradation by intracellular proteases, accumulation of metabolites at the inhibition level, are always associated with challenges [7-9]. Therefore, researchers are always trying to adopt different strategies to minimize these problems. The feeding strategy is critical to overcoming the difficulties and success of the fed-batch fermentation process because it strongly influences the required nutritional and environmental conditions for microbial growth and the overproduction of recombinant proteins [7, 10, 11]. For this purpose, various feeding strategies have been developed that have been able to successfully achieve high cell density culture (HCDC). The exponential feeding method is mostly used for HCDC of various microorganisms due to its easy application and the possibility of changing the growth rate of cells [11,12].

Studies have shown that the specific productivity of recombinant protein production in HCDC is usually less than in batch culture. Probably because most fed-batch processes are carried out at low specific growth rates and under substrate limitation in the post and pre-induction phases, and the available carbon and energy resources are mostly used for cell maintenance [8, 11, 12]. On the other hand, a higher growth rate in the production stage or after the induction of recombinant protein production increases productivity probably due to an increase of the cellular ribosome content [13], providing the appropriate substrate pre and post-induction and consequently reducing the cellular stress response. However, the implementation of the fed-batch process with a high specific growth rate is associated with challenges due to the difficulty of accurately controlling the specific growth rate. Hence, the change of the specific growth rate (as a key factor in the dynamic behavior of microorganisms in culture) within a reasonable range (considering the changes of the specific growth rate in post-induction of the optimal batch culture of the considered recombinant protein production) in a fed-batch process can provide favorable metabolic conditions for the more production of recombinant proteins [13-15].

Overexpression of recombinant proteins imposes an additional metabolic burden on the host cell and energy, carbon, and amino acid sources of cells to produce a foreign protein. As a result of the reduction of host protein synthesis, the cell growth rate reduces, and thereby the production of recombinant proteins diminishes. So, by choosing a medium rich in amino acids and induction conditions optimization can reduce the effect of metabolic load on the over-expression of recombinant proteins [16,17]. In general, defined media are used to obtain high cell densities because nutrient concentrations are known and can be controlled during cultivation. In contrast, complex media can vary in composition and quality and repeat the fermentation less. However, sometimes semi-defined or complex environments are necessary to promote cell growth and product formation. Using a defined medium with one or more amino acids or a complex compound such as yeast extract would be attractive to achieve higher cell function and/or recombinant protein [18-20].

Therefore, in this study, to obtain higher production and productivity of the LOXe enzyme, an attempt has been made to develop a suitable feeding strategy with a complex medium instead of the others reported simple medium for enhanced production of LOXe to achieve the highest

achievable growth rate in the fed-batch culture of recombinant *E. coli*. Then, by investigating the effect of glucose to yeast extract ratio on the growth kinetics of the recombinant *E. coli* and the production of LOXe enzyme, the feeding medium composition was optimized to enhance the recombinant enzyme production.

EXPERIMENTAL SECTION

Microorganism and vector system

E. coli strain Origami B (DE3) (Novagen, UK) was used as a host for LOXe expression. This strain was transformed with the recombinant plasmid pET22a (+) containing the optimized synthetic LOXe gene, which had been confirmed in our early research [15]. Protein molecular weight markers were purchased from Thermo Scientific™ (Germany). Ampicillin, IPTG, and other chemicals were acquired from Sigma (Germany). A plasmid extraction kit was obtained from Qiagen (Germany).

Media and solutions

LB (Luria–Bertani) agar medium and TB (Terrific Broth) medium were used for plate cultivation and preparation of seed culture of *E. coli* Origami B (DE3) [pET 22a (+)- *LOXe*]. The medium used as feeding for fed-batch cultivation contained glucose 200 g/L and yeast extract 200 g/L. Fed-batch cultivation was carried out in a 3.6 L bench-top stirred bioreactor (Infors AG Ch-4103, Switzerland) with a working volume of 2 L, including two six-blade Rushton impellers with a speed range of 50–1200 rpm.

Analytical procedures

Cell growth was monitored by measuring culture Optical Density (OD) at 600 nm and Dry Cell Weight (DCW). To determine DCW, 5 mL of culture medium was centrifuged at 9000 rpm for 10 min, washed twice with NaCl isotonic solution 9% (w/v), and dried at 105 °C until constant weight. Glucose and acetate were analyzed enzymatically using the appropriate kits (ChemEnzyme CO., I.R. Iran; Boehringer Mannheim/ R-Biopharm, Germany). The expression levels of LOXe were determined by SDS-PAGE using 12.5% (w/v) polyacrylamide gels. Gels were then stained with Coomassie Brilliant Blue R250 and then quantified by a gel densitometer. Total soluble protein was analyzed by the Bradford method with bovine serum albumin as a standard [21].

The stability of the plasmid in the recombinant *E. coli* strain was determined by sampling aseptically from the bioreactor at different cell densities. When required, fermentation broth samples, diluted with 9% (w/v) NaCl were plated onto LB agar plates with and without ampicillin (three replicates for each case). The fraction of plasmid-containing cells or plasmid stability was determined by calculating the ratio among the average number of colony-forming units in LB with ampicillin and without antibiotics [10,22].

The bacterial cell pellet (3 mg) was resuspended in 30 mL of lysis buffer (100 mM Tris/HCl, 5 mM EDTA, pH 7.4, and 1 mg/mL lysozyme). DNase (0.14 µL/mL) and RNase (0.14 µL/mL) enzymes were added to the lysate. The suspension was incubated for 24 hours at 4 °C. After centrifugation, the isolated pellet was washed twice with 10 mL of initial wash buffer (100 mM Tris/HCl, 50 mM NaCl, pH 7.4) and centrifuged at 11000 g for 10 min. The separated pellet was then washed with 10 mL of the second wash buffer (1% Triton x114 in the first wash buffer) and centrifuged at 11000g for 10 minutes. The isolated cell pellet was then resuspended in another 10 mL buffer (wash buffer with 1 M NaCl) and incubated for 4 h at 4 °C. The suspension was centrifuged at 11,000 g for 10 min at 4 °C. The soluble protein extract was used to measure the biological activity [23,5].

Solubilized LOXe protein was loaded onto a Hiload Sephadex 75 gel filtration column equilibrated with 10 mM Tris/HCl, 250 mM NaCl, pH 7.4 at a flow rate of 0.6 mL/min, and a wavelength of 280 nm. The protein concentration was finally determined by the Bradford method.

Enzyme activity assay with linoleic acid as Substrate

The LOXe activity of the soluble protein extract was estimated using the thiocyanate colorimetric method with some modifications. This method can measure hydroperoxides produced by lipoxygenase [1]. In this study, pure linoleic acid was used as a substrate. Linoleic acid was dissolved in 96% ethanol (10 mg/mL) (substrate solution). Then, 1 mL of the substrate solution was added to 10 mL of phosphate buffer (0.05 M, pH 7.4), followed by 0.1 mL of an aqueous solution containing LOXe (solution 1) (time 0). After 2 min and 4 min, 0.2 mL of solution 1 was removed. Then 2.5 ml of 96% ethanol was added to this solution to stop the enzyme action and

dilute the hydroperoxide concentration (solution 2). Then, 20 µL of hydrochloric acid (37 w/w%) and 2.5 µL of ammonium ferrous sulfate solution (5% w/w) in hydrochloric acid (3 w/w%) were added to solution 2 to form a color reaction (solution 3). After 30 seconds, 10 µL of ammonium thiocyanate solution (20 w/v%) was added to solution 3 and mixed. The absorbance of the final-colored solution was measured at a wavelength of 480 nm by a spectrophotometer. Results are expressed as mean values ± standard deviation (SD) in at least three independent experiments. In addition, the crude extract of *E. coli* (without enzyme production) served as a control. All measurements were performed in triplicates. All measurements were performed in triplicates.

Batch and Fed-batch cultivation

The batch culture was initially established by adding 200 ml of seed culture in a mid-exponential phase (OD_{600} = 0.7-1) to a bioreactor containing 1800 mL of the TB-modified medium (g/L) (Glucose 10, Yeast extract 24, Tryptone 12, KH_2PO_4 2.3, K_2HPO_4 12.54). The pH was maintained at 7 by adding 25% (w/v) NH_4OH or 3 M H_3PO_4. Dissolved oxygen was maintained by controlling the inlet air (enriched with pure oxygen) and agitation rate at 30-40% air saturation. The total gas flow rate (air + pure oxygen) was kept at 2vvm. The foam was controlled by adding a silicone antifoam reagent. In batch culture, induction was performed by adding an inducer in OD_{600}= 4.5 ± 0.2, and this process continued until growth stopped.

In all fed-batch fermentation processes, the batch stage was continued until the initial glucose ran out in the medium. After the depletion of initial glucose in the medium, as indicated by a rapid increase in the dissolved oxygen concentration, feeding was initiated. The feeding rate was increased based on the exponential feeding strategy with the maximum achievable specific growth rate during fed-batch cultivation. The exponential feeding was determined by the following equation (Eq. (1)) [8,11]:

$$Ms(t) = F(t)S_0 = [\mu / Y_{x/s} + m]X_0S_0V_0 \exp(\int_{t0}^{t} \mu(t)dt) \qquad (1)$$

Where V_0 is the volume of the medium in the bioreactor (L), X_0 is the biomass concentration at the start of feeding g(DCW)/L, t the time (h), μ the specific growth rate (1/h), S_0 is the glucose concentration (g/L) in the feeding solution, $F(t)$ the feeding rate (h^{-1}), $M(t)$ the mass feeding rate (g/h), $Y_{X/S}$ the yield of biomass as a result of the substrate (g DCW /g glucose), t_0 (h) is the starting time for each feeding step, and m is the specific maintenance coefficient (g/(g.h)). The yield ($Y_{X/S}$) and maintenance coefficient (m) were set at 1 and 0.025 g/(g.h), respectively [8, 11].

To develop a simple feeding strategy with the highest achievable specific growth rate at all stages of the fermentation process, the maximum oxygen transfer capacity of the bioreactor was used. The maximum oxygen transfer capacity is provided by increasing the stirring speed to the maximum possible value of 1100 rpm and the aeration speed to 2 vvm and increasing the oxygen content of the inlet air to 100%. Of course, this increases in proportion to the amount of biomass and specific growth rate, keeping the oxygen concentration above 20% saturation. However, when these conditions were established and the oxygen concentration began to drop below 10%, to control the oxygen concentration, the specific growth rate was reduced over time by decreasing the feeding rate until it stopped at zero at the end of the process.

Glucose concentration was maintained below 2 g/L by gradually increasing feeding in each stage. With the increase in feeding rate, stirring rate, and the oxygen percentage of the inlet air increased to control the oxygen concentration. In all experiments, cells were induced by adding 0.2 mM IPTG.

The required nitrogen source (ammonium) was supplied by the addition of 25% (w/v) NH_4OH, which was also used to maintain the pH at 7. The level of phosphate added at the beginning of the fed batch was sufficient to last the entire process. The glucose concentration was controlled manually at 10-minute intervals by their monitoring.

RESULT AND DISCUSSION

There are several reports that fed-batch cultures with exponential feeding with high specific growth rates have been used to overproduce various recombinant proteins. The amount of recombinant protein produced in these processes depends on various factors, including the type of microorganism, the type of recombinant protein, the composition of the medium, and the limitations of the bioreactors used for the research.

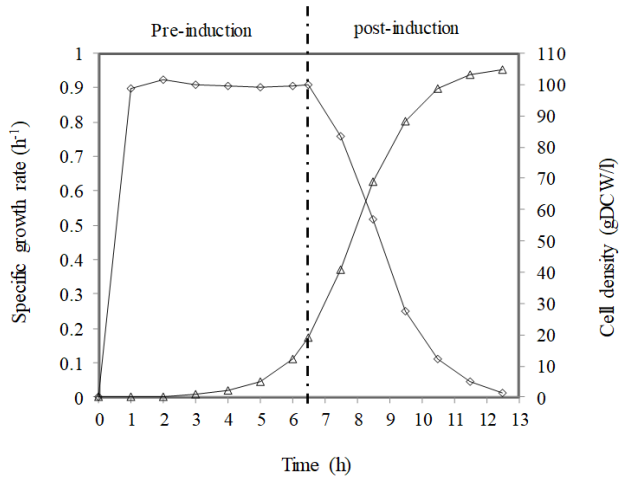

Fig. 1: Effect feeding strategy with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction in fed-batch cultures E. coli BL21 (DE3) [pET21a (+) - LOXe] by IPTG 0.3 mM and feeding medium containing glucose 200 g/L and yeast extract 200 g/L. Specific growth rate (h^{-1}) (◊); and cell dry weight (g/L) (Δ)

Successful research by *Babaeipour et al*. on the production increase of γ-interferon and Granulocyte Colony-Stimulating Factor (GCSF) showed that the feeding strategy with the maximum achievable specific growth rate before and after induction significantly increases the *E. coli* growth and the recombinant protein production in comparison with other feeding methods [8,10,23]. Therefore, for the first time, this study attempted to develop a fed-batch culture with a constant maximum specific growth rate before induction and a variable specific growth rate with a maximum achievable after induction with a complex feed instead of a defined medium to production enhancement LOXe. Besides, by optimization of the medium composition and the amount of inducer, the production of recombinant LOXe was increased.

For this purpose, it was hypothesized that feeding could be done in such a way that, similar to the optimized batch culture, the specific growth rate was maintained at a maximum of 0.92 ± 0.2 h^{-1} before induction (Fig. 1) But after induction, specific growth rate changes similar to its post-induction changes in an optimal batch culture obtained in the previous research [15] that during the period 5.5 ± 0.5 hours, it diminishes of 0.92 ± 0.2 h^{-1} to less than 0.05 at the end of the process due to stopped cell growth and recombinant protein production.

Based on this, it was calculated that if the cell density (OD_{600}) at the induction time be 40 (dry weight about 19 g/L), the final biomass concentration will be more than 100 g/L, which is one of the highest value ever reported. Therefore, the time to achieve cell density OD_{600} = 40 was chosen as the appropriate time to induce recombinant LOXe production. To perform such experiments, the feeding rate increased to maintain the glucose concentration within a permissible range, and at the same time, the maximum oxygen transfer capacity of the bioreactor was used. Therefore, during this process, oxygen concentrations higher than 20% of saturation, and glucose levels greater than zero and less than the allowed amount (2 g/L) were maintained.

Figs. 1 to 3 show the results of using this feed strategy for the fed-batch culture of *E. coli* BL21 (DE3) [pET21a (+) - LOXe]. It is observed that using a feed strategy with a variable growth rate at the maximum achievable amount can result in higher cell density and consequently higher specific yield of LOXe production. Fig.-1 shows that 1) bacterial growth and recombinant protein production are stopped at the same time, 2) the time of the fed-batch fermentation process is less than 13 hours, which is the fastest growth that has been reported for the production of recombinant protein by the fed-batch fermentation method, 3) After induction, the specific growth rate decreased rapidly due to the high metabolic load resulting from the high expression of rLOXe. It has already been shown that the simultaneous achievement of the highest amount of biomass and the production of recombinant protein provides the possibility of achieving the highest amount of production.

Fig. 2 shows comparison of LOXe recombinant protein production in optimal batch culture (induction at OD600 =5) and fed-batch culture (with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction) induced at OD600 =40 by IPTG 0.5 mM, and feeding medium containing glucose 200 g/L and yeast extract 200 g/L. This Fig. also shows that the used feed strategy can maintain a high amount of recombinant enzyme expression. Also, Fig. 2 shows that the stability of the plasmid in the developed Fedbatch culture is maintained above 95% until the end of the process. Reducing the time of the fermentation process obtained by using the feeding strategy with the maximum specific growth rate before and

Table 1: Comparison results of optimum batch culture in complex TB medium and fed-batch culture with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction E. coli BL21 (DE3) [pET21a (+)-LOXe] induced by IPTG 0.5 mM at OD_{600} =40 and feeding media containing 200 g/l glucose and yeast extract 200 g/l.

Factors	Fed-batch	Batch (with complex media)
Fermentation time (h)	12.5 ± 0.5	9.75 ± 0.25
Amount of consumed glucose (g)	55	10
Final cell density (g CDW/l)	104 ± 2. 5	20.15 ± 0. 5
biomass productivity (gCDW/l.h)	8.16 ± 0.2	2.07 ± 0.10
Final rLOXe concentration (g/l)	27.35 ± 0.4	5.14 ± 0.15
Biomass specific yield, Yx/s (g CDW/g glucose)	1.9 ± 0.1	2.5 ± 0.1
rLOXe production specific yield, Yp/s (g rLOXe /g glucose)	263 ± 10	255 ± 10
rLOXe productivity (g rLOXe /l.h)	2.19 ± 0.03	0.527 ± 0.02

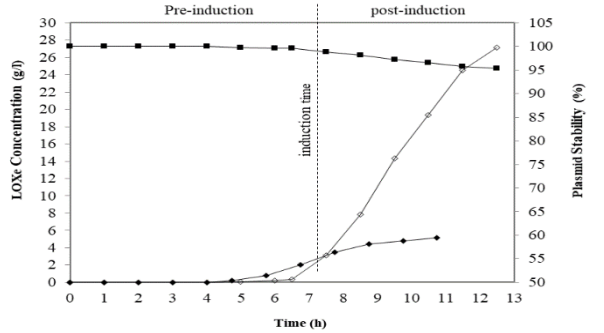

Fig. 2: Plasmid stability and rLOXe production in fed-batch culture (with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction) E. coli BL21 (DE3) [pET21a (+) - LOXe] induced at OD_{600} =40 by IPTG 0.5 mM, and feeding medium containing glucose 200 g/L and yeast extract 200 g/L and rLOXe production in optimal batch culture (induction at OD_{600} =5) and Amount of rLOXe production in batch culture (♦) and fed-batch culture (◊) and plasmid stability profile in this fed-batch culture (■).

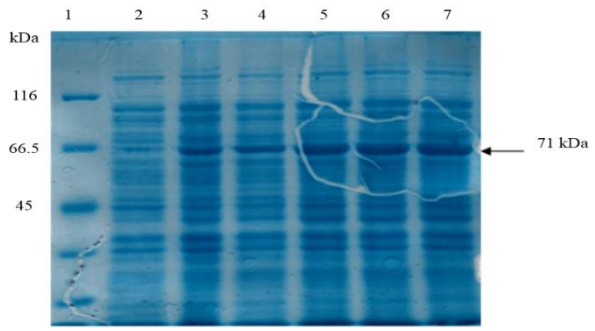

Fig. 3: Gel electrophoresis expression of lipoxygenase in fed-batch culture with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction E. coli BL21 (DE3) [pET21a (+) - LOXe] induced by IPTG 0.5 mM at OD_{600} =40 and feeding media containing 200 g/L glucose and yeast extract 200 g/L column 1) Molecular weight marker, 2) Sample Pre-induction, 3–7) 2 6 h after induction, respectively

after the induction had a great effect on the preservation of the plasmid of the recombinant strain. This is in agreement with results reported using the same strategy for the production of interferon-gamma and GCSF [10,23]. Fig. 3 indicates the expression of lipoxygenase at different times in batch culture of *E. coli* BL21 (DE3) [pET21a (+)-LOXe]. It can be found that the lipoxygenase expression and cell density did not change significantly 4 hours and 5 hours after induction, respectively. Therefore, following the research steps, the induction continued up to 5 hours after inducer adding, as the optimal time for the production phase.

The results of this study and its comparison with the optimal results of batch culture have been summarized in Table 1. It can be seen that with the development of fed-batch culture with the constant maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction of recombinant protein production, only with a 3-hour increase in fermentation time with an increase of more than 5-fold increase from 5.14 reaches 27.35 g/L. Total rLOXe production also increased from 0.527 to 2.19 g/L, which is one of the highest values reported values for recombinant protein production. Meanwhile, the overall efficiency of biomass production and rLOXe production increased by 4 times to 2.19 ± 0.03 gCDW/L.h and 8.16 ± 0.2 g rLOXe /L.h, respectively. These values are among the highest values reported so far for recombinant *E. coli*.

Effect of feeding media composition of fed-batch culture on rLOXe production

Many researchers have reported the use of the combination of yeast extract and glucose in the feeding medium culture for the over-production of various recombinant

Table 2: Summary of results of fed-batch culture with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction E. coli BL21 (DE3) [pET21a (+) - LOXe] using different amounts of glucose and yeast extract in the feeding medium

Factors	Glucose 200 g/l Yeast Extract 25 g/l	Glucose 200 g/l Yeast Extract 50 g/l	Glucose 200 g/l Yeast Extract 100 g/l	Glucose 200 g/l Yeast Extract 200 g/l
Fermentation time (h)	14.5 ± 0.5	13.5 ± 0.5	12.5 ± 0.5	12.5 ± 0.5
Amount of consumed glucose (g)	120	95	70	55
Final cell density (g CDW/l)	90 ± 2.5	99 ± 2	109 ± 2.5	104 ± 2.5
biomass productivity (gCDW/l.h)	6.21 ± 0.2	7.33 ± 0.2	8.72 ± 0.2	8.16 ± 0.2
Final rLOXe concentration (g/l)	19.82 ± 0.3	23.15 ± 0.3	30.43 ± 0.4	27.35 ± 0.4
Biomass specific yield, Yx/s (g CDW/g glucose)	0.75 ± 0.1	1.04 ± 0.1	1.58 ± 0.1	2.17 ± 0.1
rLOXe production specific yield, Yp/s (g rLOXe /g glucose)	220 ± 10	234 ± 10	278 ± 10	263 ± 10
rLOXe productivity (g rLOXe /l.h)	1.37 ± 0.1	1.72 ± 0.1	2.43 ± 0.1	2.19 ± 0.03

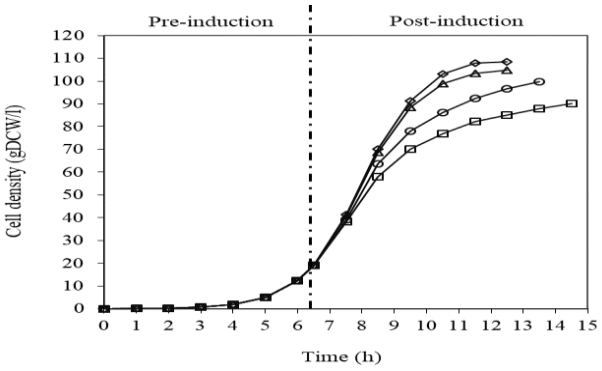

Fig. 4: Effect of feeding media composition on growth of E. coli BL21 (DE3) [pET21a (+) - LOXe] in fed-batch culture with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction. glucose 200 g/L and YE 200 g/L (Δ), YE 100 g/L (◊), YE 50 g/L (○), and YE 25 g/L (□)

proteins in the batch processes [8,20,25]. However, due to the difficulty of controlling the growth rate by feeding with a complex culture medium, it has not been used in Fed-batch culture [26, 27]. On the other hand, different ratios of yeast extract and glucose have been used to overproduce different proteins. Hence, the overproduction of recombinant LOXe requires optimization of the glucose-to-yeast extract ratio, which stimulates bacterial growth to further increase production. Therefore, in this part of the study, the effect of glucose to yeast extract ratio in four ratios of 1, 2, 4, and 8 on bacterial growth kinetics and recombinant protein production was investigated. The results have been shown in Figs. 4, 5, and 6 and Table 2.

Fig. 4 indicates that by increasing the ratio of glucose to yeast extract from 1 to 2, the amount of biomass produced increases from 100 to 105 g/L. However, with a further increase in the ratio of glucose to yeast extract due to glucose accumulation (as shown in Fig. 7), acetate is produced and accumulated as the main metabolic byproduct of *E. coli*, resulting in the final amount of the biomass reduced. Acetate has a great inhibitory effect on the growth of bacteria and the production of recombinant proteins, and depending on the amount of acetate produced, their amount decreases significantly. Several reports have shown that a concentration of more than 3 g/L of acetate in fed-batch culture media reduces bacterial growth and recombinant protein production.

Fig. 5 shows that at the concentration of 200 g/L of glucose in the feed, by reducing the amount of yeast extract from 200 g to 25 g/L, there was no significant change in the growth rate before induction. But after induction, each it has been closer to the end of the process, the growth rate has decreased faster, and subsequently, the fed-batch process time has increased.

Fig. 6 shows the effect of feeding media composition on rLOXe production in specific growth rate in fed-batch culture of *E. coli* BL21 (DE3) [pET21a (+) - *LOXe*] with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction. Fig. 6 indicates that by increasing the ratio of glucose to yeast extract from 1 to 2, the amount of rLOXe produced increases from 27.35 to 30.43 g/L. However, as the ratio of glucose to yeast extract further increases due to glucose accumulation (as shown in Fig. 7), acetate is produced and accumulated as the main metabolic byproduct of *E. coli*. Acetate has a great inhibitory effect on the growth of bacteria and the production of recombinant

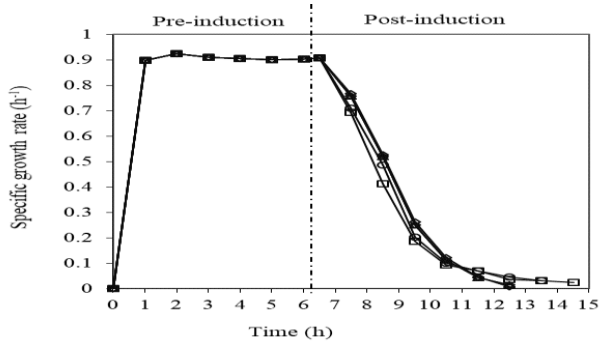

Fig. 5: Effect of feeding media composition on specific growth rate of E. coli BL21 (DE3) [pET21a (+)-LOXe] in fed-batch culture with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction. glucose 200 g/L and YE 200 g/L (Δ), YE 100 g/L (◊), YE 50 g/L (○), and YE 25 g/L (□)

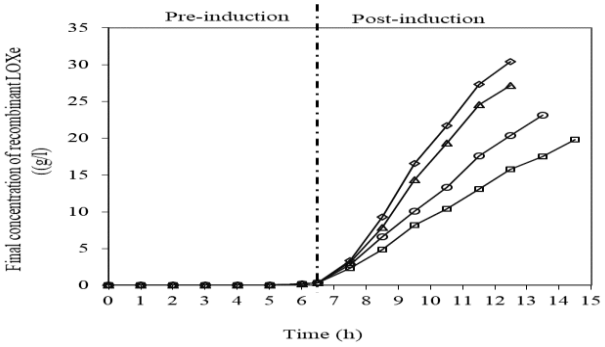

Fig. 6: Effect of feeding media composition on rLOXe production in specific growth rate in fed-batch culture of E. coli BL21 (DE3) [pET21a (+) - LOXe] with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction. glucose 200 g/L and YE 200 g/L (Δ), YE 100 g/L (◊), YE 50 g/L (○), and YE 25 g/L (□)

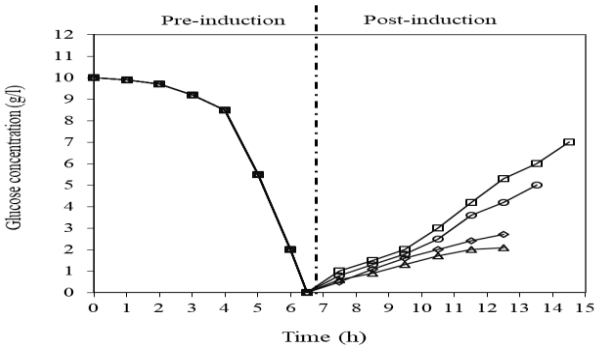

Fig. 7: Effect of feeding media composition on glucose concentration of E. coli BL21 (DE3) [pET21a (+)-LOXe] in fed-batch culture with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction. glucose 200 g/L and YE 200 g/L (Δ), YE 100 g/L (◊), YE 50 g/L (○), and YE 25 g/L (□)

proteins, and depending on the amount of acetate produced, their amount decreases significantly. Several reports have shown that a concentration of more than 3 g/L of acetate in fed-batch culture media reduces bacterial growth and recombinant protein production.

The use of simple culture media with specific chemical composition compared to complex media usually reduces the amount and rate of bacterial growth and recombinant protein production [20,27,28]. However, using the defined culture medium, it is easier and better to control the concentration of media culture compounds and the fermentation process, and also the purification of extracellular recombinant protein is facilitated. On the other hand, complex and semi-defined culture media significantly increase the amount and rate of recombinant protein production compared to defined culture media due to the growth factors they have.

It has been shown that the addition of yeast extract to the culture media reduces the production of toxic metabolites such as acetic acid and helps the uptake of acetate by the cells in the absence of the main carbon source [24, 25]. It has also been reported that the use of an organic nitrogen source similar to yeast extract increases the productivity of recombinant protein, especially in high cell density cultures, which require a much higher nitrogen supply after induction of protein expression [29].

It was expected that the use of yeast extract with glucose would achieve a higher growth rate during the fermentation process and produce less acetate. The presence of yeast extract in the culture medium provides the required precursors for the synthesis of essential cell components and therefore more glucose is used as an energy source [20,29,30]. Also, the presence of yeast extract reduces the inhibitory effects of acetic acid and as a physiological buffer, controls the pH of the culture medium better than the defined medium [29,31]. In general, this study indicates that the use of yeast extract with glucose increases the productivity of recombinant protein and the specific growth rate of *E. coli* and reduces production time, resulting in a significant increase in the overall productivity of protein production.

Effect of inducer on cell growth and LOXe recombinant enzyme production

In previous experiments, IPTG 0.5 mmol/L was used to induce LOXe recombinant enzyme production in fed-batch

Table 3: Comparison of the results before and after optimization of inducer value in fed-batch culture with fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction E. coli BL21 (DE3) [pET21a (+)-LOXe] with and feeding media containing 200 g/l glucose and yeast extract 100 g/l.

Factors	Before optimization 4 mg(IPTG)/(g (DCW).L)	After optimization 6 mg(IPTG)/(g (DCW).L)
Fermentation time (h)	13.5 ± 0.5	12.5 ± 0.5
Amount of consumed glucose (g)	75	70
Final cell density (g CDW/l)	119 ± 2.5	109 ± 2.5
Biomass overall productivity (gCDW/l.h)	8.82 ± 0.2	8.72 ± 0.2
Final rLOXe concentration (g/l)	36.45 ± 0.4	30.43 ± 0.4
Biomass specific yield, Yx/s (g CDW/g glucose)	1.59 ± 0.1	1.58 ± 0.1
rLOXe production specific yield, Yp/s (g rLOXe /g glucose)	306 ± 10	278 ± 10
rLOXe productivity (g rLOXe /l.h)	2.71 ± 0.1	2.43 ± 0.1

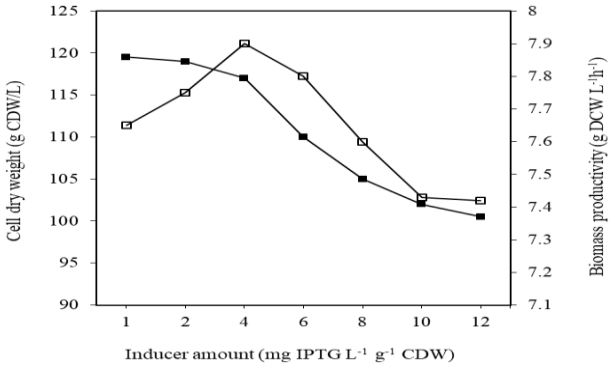

Fig. 8: Effect of inducer value (g IPTG $L^{-1}.g^{-1}$ DCW) on final cell density (g DCW /L) (■) and overall productivity of biomass (g DCW $L^{-1}h^{-1}$) (Ɣ) in fed-batch culture of E. coli BL21 (DE3) [pET21a (+)-LOXe]

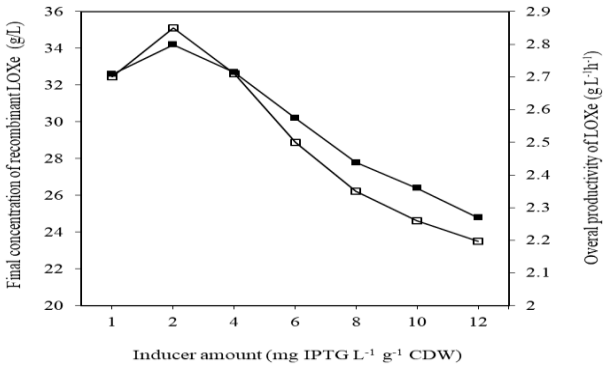

Fig. 9: Effect of inducer amount (g IPTG L^{-1} g^{-1} DCW) on the final production of LOXe (g LOXe /L) (1) and overall productivity of recombinant LOXe (g LOXe $L^{-1}h^{-1}$)) in fed-batch culture E. coli BL21 (DE3) [pET21a (+)-LOXe]

culture. Although the IPTG value for expressing *Lac* promoter systems is often given in mmol/L, to quantify the effect of cell density on the inducer value, it was first necessary to calculate the used IPTG value in these experiments based on cell dry weight instead of volume. The calculated value of 4 mg (IPTG)/(g (DCW).L) from previous experiments in batch and batch cultures was used as the reference point for selecting different inducer values.

Figs. 8 and 9 show the effect of the amount of inducer on cell growth and rLOXe enzyme production in fed-batch culture with *E. coli* BL21 (DE3) [pET21a (+)-LOXe] induced at OD_{600}=40. From Figs. 8 and 9, it can be seen that the final cell density and overall productivity of the biomass increases with the reduction of the amount of inducer from 4 to 12 mg/(g.L). As the inducer was further reduced, up to 1 mg/(g.L), the final cell density remained constant, but the overall productivity of biomass declined due to increased fermentation time. Fig. 9 shows that the final concentration and overall productivity of recombinant LOXe increases with decreasing inducer concentration from 12 to 2 mg/(g.L), but decreases with a further reduction of inducer up to 1 mg/(g.L).

Comparison of the results before and after optimization of inducer value in fed-batch culture with the fixed maximum specific growth rate in pre-induction and variable maximum attainable specific growth rate in post-induction *E. coli* BL21 (DE3) [pET21a (+)-LOXe] with and feeding media containing 200 g/L glucose and yeast extract 200 g/L presented in table 3. It can be found that the optimization inducer amount increases by about 20% rLOXe enzyme production.

To better understand the relationship between cell growth and rLOXe production with inducer value, the specific growth rate and specific rLOXe production rate (q_p) curves were plotted against changes in inducer value (Fig. 10). It is observed that changes in the specific growth rate after induction decrease for all higher values of the

Table 4. the comparison activity of LOXe crude extract E. coli with and without LOXe

Reaction mixture	Reaction time (min)	Absorbance
Crude *E. coli* extract with LOXe	0	0.431±0.01
	2	0.652±0.02
	4	0.882±0.01
Crude *E. coli* extract without LOXe	0	0.441±0.03
	2	0.441±0.01
	4	0.431±0.02

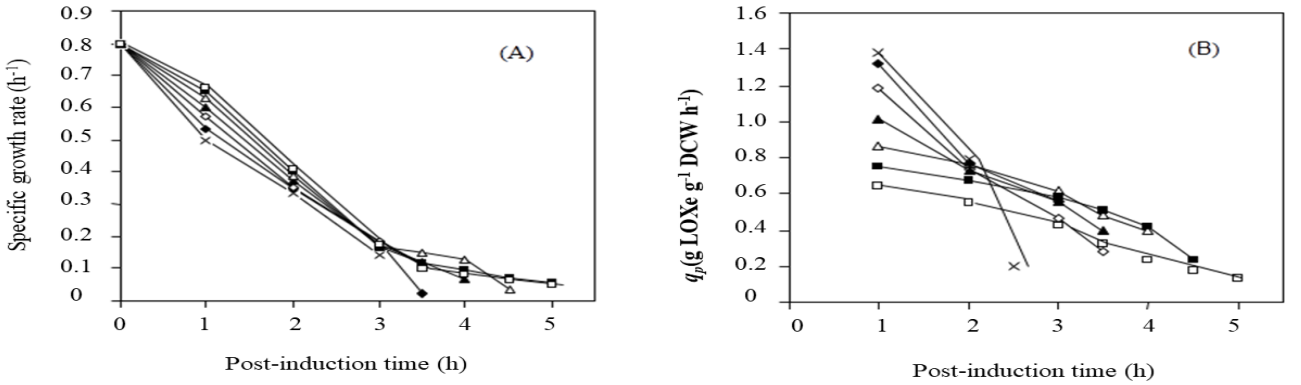

Fig. 10: Time profile: (a) specific growth rate (μ), (b) LOXe recombinant protein production rate (q_p) in various amounts of inducer (g IPTG L^{-1} g^{-1} DCW): 12 (×), 10 (♦), 8 (◊), 6 (▲), 4 (Δ), 2 (■), 1 (□)

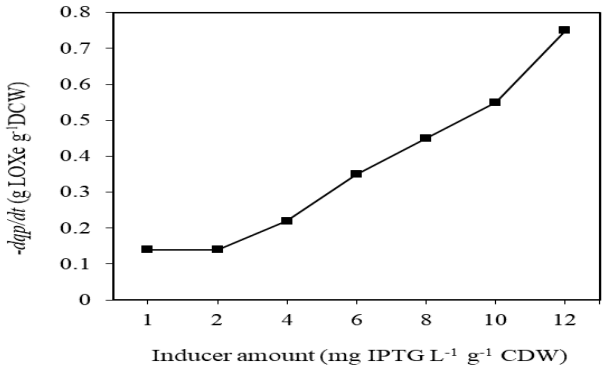

Fig. 11: Time profile of LOXe recombinant enzyme production rate relative to the inducer amount in fed-batch culture E. coli BL21 (DE3) [pET21a (+) - LOXe]

inducer IPTG, especially in the three hours after induction (Fig. 10A). The specific rate of rLOXe enzyme production also decreases sharply after induction (Fig. 10B). Fig. 11 indicates the time profile of LOXe recombinant enzyme production rate relative to the inducer amount in fed-batch culture *E. coli* BL21 (DE3) [pET21a (+) - *LOXe*]. It can be found that the deceleration rate q_p ($-d(q_p)/dt$) is also directly proportional to the amount of IPTG (Fig. 11).

In general, rates of cell growth and LOXe production decrease with increasing IPTG content. The results of the inducer effect exhibit that there is no direct relationship between the inducer amount and the expression level of recombinant protein. In fact, the amount of recombinant protein production in fed-batch culture combined with feeding is a steady exchange between the biomass yield and rLOXe production rate. As a result, moderate amounts of 2-4 mg (IPTG) g^{-1} (DCW) L^{-1} of inducer achieve the maximum cell growth and rLOXe over-production simultaneously. Therefore, 2mg (IPTG) g^{-1} (DCW) L^{-1} was selected as the optimum inducer for a high yield of rLOXe.

Determination of LOXe activity

LOXe catalyzes the oxygenation of polyunsaturated fatty acids such as linoleic acid to conjugated unsaturated fatty acid hydroperoxides. The activity of LOXe was measured by crude extract of the LOXe from *E. coli* using the ferric thiocyanate method. In this method, LOXe-derived unsaturated fatty acid hydroperoxides oxidize the Fe^{2+} ion to the Fe^{3+} ion. The Fe^{3+} binds with thiocyanate and produces a red ferric thiocyanate (FTC) complex. The absorbance of the FTC complex is measured at 480 nm. Table 4 compares the activity of LOXe crude extract *E. coli* with and without LOXe in the optimized fed-batch culture *E. coli* BL21 (DE3) [pET21a (+) - LOXe].

CONCLUSIONS

Fed-batch fermentation processes of recombinant *E. coli* due to the inability to properly control growth rate, high rate of CO_2, heat production, high demand, and limited oxygen transport capacity of oxygen, generally are performed under conservative conditions such as using a simple culture medium or feeding based on a constant specific growth rate to minimize the challenges ahead. Implementation of fed-batch processes under a low specific growth rate and the limited substrate amount before and after induction increases cell stress response and consequently reduces maximum attainable cell growth and recombinant protein production. In this study, for the first time, to achieve a higher production amount and productivity of the rLOXe enzyme, a suitable feeding strategy was developed to achieve the highest growth rate achievable in the fed-batch culture of recombinant *E. coli* using a complex feeding medium. Then, by examining the effect of glucose and yeast extract ratio and then the amount of inducer on the growth kinetics of *E. coli* and LOXe production, and optimization of feeding medium composition was increased the r-LOXe production. Using the developed feeding strategy, 36.4 g/L rLOXe was obtained with an overall productivity of 2.71 $g.l^{-1}.h^{-1}$. This is one of the highest productivity ever has been reported for the recombinant protein production of *E. coli*. It is expected that this strategy can be successfully used for the overproduction of other recombinant proteins and the high cell densities of various microorganisms with maximum achievable productivity. Therefore, in future research, we plan to use the developed Fed-Batch strategy to produce many more recombinant products from Escherichia coli, and also, solve the problems of its implementation on a higher scale, by examining the effect of the scale-up of the process on its performance.

Abbreviation

LOXs	Lipoxygenases
AmbL OXe	Amphibian epidermal lipoxygenase
HCDC	High Cell Density Culture
LB	Luria–Bertani
TB	Terrific Broth
OD_{600}	optical density at 600 nm
IPTG	Isopropyl β- d-1-thiogalactopyranoside
GCSF	Granulocyte colony-stimulating factor
vvm	Air volume per culture medium volume per minute
DCW	Dry cell weight

Acknowledgment

The authors wish to thank the management department of Bioscience and Biotechnology Malek Ashtar University of Technology, School of Pharmacy and Bioinformatics Research Center, Isfahan University of Medical Sciences, and Faculty of Pharmacy, Shahid Beheshti University of Medical Science for providing the necessary conditions for carrying out this research.

Received : Mar.03, 2023 ; Accepted : Jul.10, 2023

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