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Research Article

# Acetate Kinase a Antisense Delivery by PAMAM Dendrimer for Decreasing Acetate Production and Increasing the Production of Recombinant Albumin in *E. coli*

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**Background:** Acetate accumulation in the culture medium is known as an inhibitor in recombinant protein production in *Escherichia coli*. Various approaches have been proposed and evaluated to overcome this challenge and reduce the concentration of acetate. In this study, we examined the effect of acetate kinase A antisense on acetate production rate in *E. coli* We also used PAMAM dendrimers as a suitable delivery agent for antisense transformation into *E. coli* host cell.
**Objective:** This study aimed to decrease acetate production as a by-product using an antisense-dendrimer complex to increase mass cell and subsequently recombinant Albumin production in *E. coli*.
**Materials and Methods:** Here, to study the effect of this treatment on recombinant protein production, we used pET22b/HAS construct. The ackA gene expression was inhibited by designed antisense to reduce acetate concentration in culture medium. AckA antisense was transferred to *E. coli* by PAMAM dendrimer. Finally, ackA expression and recombinant Albumin production were evaluated Real-Time PCR and densitometry, respectively.
**Results:** Our data showed, designed antisense lead to reduction of acetate kinase gene expression and subsequently acetate concentration in the culture medium. Finally, acetate concentration reduction and cell mass increase result in enhanced recombinant Alb production in the treated group (1.25 $mg.mL^{-1}$) compare to the control group ( 0.59 $mg.mL^{-1}$).
**Conclusions:** Reduction of acetate in *E. coli* fermentation process decreased the recombinant Alb production following cell growth and cell mass increase. In the current study, we showed that an antisense can be a useful tool for ackA gene expression reduction. Also, we noted that PAMAM dendrimer could be a proper delivery agent for oligonucleotide antisense transformation into bacterial cells.

*Keywords*: Acetate, Acetate kinase A, Antisense, PAMAM dendrimer, Recombinant albumin

## 1. Background

Human serum albumin (HSA), the most abundant protein in the circulatory system, transports several metabolites and hormones in the body and plays key roles in blood volume stabilization and burn treatment (1). Production of a higher amount of useful proteins like HSA, per unit volume per unit time, is the final goal of recombinant proteins' industrial production (2). Optimization of critical parameters like culture medium, fermentation process condition, and modifying the host organism affect the final product yields (3). *Escherichia coli* is the first option for producing many recombinant proteins because this organism is user-friendly and has low manufacturing costs. For *E. coli* as an expression system, final cell density directly affects final recombinant protein yield (4). Production of acetic acid (acetate) as an undesirable extracellular by-product is one of the most important disadvantages of *E. coli*. Acetate kinase (encoded by ackA gene) has the main role in acetate production in *E. coli*.

As a major metabolite of glucose metabolism, acetic acid has several negative effects on cell density and recombinant protein concentration. Several strategies reduce acetic acid accumulation in culture media and its negative effects on recombinant protein concentration (5). These strategies include "knockout" mutations for blocking the acetate production, controlling medium ingredients like carbon sources (e.g., glucose) to reduce the burden on the glycolysis path. Although acetate has a negative effect on productivity, it can be a source of ATP formation in *E. coli* under anaerobic and even aerobic conditions. The use of antisense oligonucleotide technology provided an approach to reduce acetate expression moderately so that it still maintained the useful functions of this pathway (6). Antisense oligonucleotides (ASOs), synthetic 15-25 nt oligodeoxynucleotides, can bind to RNA and reduce or regulate gene expression via several mechanisms (7).
ASO attaches to the target mRNA and recruits RNase H to cleave and degrade the mRNA, thereby lowering protein levels. Also, ASO can inhibit translation by block the translation machinery in the ribosome site (8). The key problem for oligonucleotide-based gene downregulation is to deliver the active oligonucleotide to the cells. Various delivery systems, including cationic polymers, liposomes, and different polymeric nanoparticles, have been employed to deliver antisense (9). In recent years, dendrimers' use as a delivery system in medical and biological fields has been increased. Characteristics such as structural properties (highly branched globular structures, uniformity, and multivalency), variable chemical composition, and high biocompatibility cause dendrimers to be interesting for scientists (10). The present study intends to evaluate the effects of ASO delivered by the dendrimer system on decreasing the acetate and increasing the production of rh-Albumin in *E. coli.*

## 2. Objectives

This study aimed to decrease acetate production as a by-product by an antisense-dendrimer complex to increase mass cell and recombinant Albumin production in *E. coli*.

## 3. Materials and Methods

### *3.1. Materials*

Polyamidoamine (PAMAM) dendrimers were purchased from Dendritech (Dendritech Inc, MI) and were applied without further purification. This material has an initiator core that is an ethylenediamine molecule. The plasmid construction (pET22b/HSA) expressing the *human serum albumin* gene was a generous gift of Dr. Bahram Kazemi Lab (Department of Biotechnology, Shahid Beheshti University of medical sciences). *Escherichia coli* BL21 (DE3) was the chosen host. 2X Real-Time PCR Master Mix (For SYBR Green I) was purchased from BioFACT company. RevertAid First Strand cDNA Synthesis Kit purchased from Thermo Scientific Company for cDNA synthesis from RNA.

### *3.2. Antisense Oligonucleotide*

A phosphorothioate 2′-O-methyl-oligonucleotide (5'ATTTCAGTGAAGAACTACCGC 3′) was designed complementary in the coding region of ackA mRNA. The fully phosphorothioated antisense oligonucleotide was synthesized by Aoke Biotechnology Co., Ltd. (Beijing, China).

### *3.3. Western Blot Analysis of Recombinant Albumin*

BL21(DE3) *E. coli* transformed with pET22b/HSA, was cultured in Luria–Bertani medium with 100 $\mu g.mL^{-1}$ ampicillin at 37 °C at 150 rpm. When the OD600 of the bacterial culture reached 0.6, the Isopropyl-D-1-thiogalactopyranoside (IPTG) (Merck, Germany) was employed with a final concentration 0.5 mM as an inducer. After 4 hours of IPTG-induction, the bacterial cells were harvested, and then for the analysis, the cells were treated with lysis buffer (Tris 50 mM, 10% glycerol, 0.1% Triton X-100) (Merck, Germany) (11). The analysis of the cell lysate was done by the SDS-PAGE gel 12.5%, 2 h at 100 V, along with molecular weight markers (Sigma). The expression of albumin was confirmed by western blotting. The resolved proteins were transferred to a nitrocellulose membrane (Wathman, UK), and the membrane was blocked using Phosphate buffered saline with 5% nonfat milk. The blocked membrane was then immersed in a 1:2000 dilution of peroxidase-conjugated anti-His-tag monoclonal antibody (Abcam, UK) for 2 hours at room temperature. The immunoreactivity was visualized using chromogenic substrate 3, 3'-diaminobenzidine (DAB) (Roche, Germany) (12).

### *3.4. Preparation of ASO Complexes with PAMAM*

Dendrimers were diluted appropriately in manufacturer buffer, and all solutions were stored at 4 °C. Antisense–dendrimer complexes were obtained by incubating antisense oligonucleotides and dendrimers together in 100 µL water at room temperature for 5 min. The electrostatic charge on each component is an essential factor in the calculation of ratios for dendrimer-antisense complexes. These values are obtained by comparing the number of phosphate groups of antisense

**Table 1**. Ratios for dendrimer - antisense complex for analyzing by electrophoresis

| *N:P Ratio in dendrimer - antisense complex* | *Dendrimer in complex ( µg)* | *Antisense in complex ( µg)* |
|---|---|---|
| **1:1** | 0.65 | 1 |
| **2:1** | 1.3 | 1 |
| **3:1** | 2.6 | 1 |
| **1:2** | 0.65 | 2 |
| ***Control*** | - | *1* |

and NH2 groups of the dendrimer. According to the manufacturer, about $1.71 \times 10^{15}$ negative charges are present per 1 µg ASO while a G5 (NH 3) dendrimer has $\sim 2.65 \times 10^{15}$ charges per microgram. So, 1 µg of antisense has an equal number of charges with 0.65 µg of dendrimer (1:1 charge ratio). Different Dendrimer/ASO charge ratios were prepared and studied for complex formation potential in the current study. (**Table 1**). After pipetting (about15 times) and incubating for 20 min, the complexes were evaluated by electrophoresis on a 4% agarose gel stained with ethidium bromide and run for one hour at 90 V and finally visualized using a UVP gel documentation system (13).

*3.5. Transformation of E. coli Using PAMAM/ASO Complex*

BL21 cells were transformed by mixing the PAMAM/ASO complex and 1 mL of the cells in LB media for 4 hours at 37 °C with gentle shaking. LB media containing 1.5% agar were used for plating the mixtures. The incubation was then performed at 37 °C for 17 h (14).

*3.6. Cytotoxicity Assay*

To analyze PAMAM, PAMAM-ASO, and ASO's cytotoxicity effect, we used Minimum Inhibitory Concentration (MIC) assays using a microdilution method represented by the Clinical Laboratory Standards Institute (15).

In this method, briefly, 2-fold serial dilutions of the dendrimer, ASO-dendrimer, and ASO were provided in sterile Mueller Hinton Broth (MHB) (dilution ratio range: 1:1 to 1:256). Each well of a microtiter plate was coated with 100 µL from each dilution, and then the plate was incubated with 5 µL of standardized bacterial suspension ($1.5 \times 10^7$ CFU.mL$^{-1}$). After aerobic incubation at 37 °C for 16 h, the cultures were investigated for bacterial growth as turbidity. The lowest concentration of agents (dendrimer, ASO-dendrimer, and ASO) that prevented the visible bacterial growth was determined to be the MIC. One well was considered as a positive control (only media, inoculum, no PAMAM, PAMAM-ASO, and ASO) for all tested samples, and another well was considered as a negative control (only media, no inoculum, no PAMAM, PAMAM-ASO, and ASO) (16).

*3.7. RNA Isolation and Reverse Transcription*

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the instructions of the manufacturer. To proliferation of the ackA gene, the cDNA of ackA was synthesized by reverse transcription PCR (RT-PCR) from 1 µg of each RNA sample. The first strand of cDNA was synthesized from 1 µg of total RNA with a final volume of 20 µL, using SuperScript II (Invitrogen, Carlsbad) and oligo(dT) primers. Then, PCR amplification was done by an Eppendorf AG 22331, Germany. The cDNA was stored at 20 ºC.

*3.8. Quantitative Real-time PCR Reaction*

The synthesized cDNA was amplified using real-time PCR (StepOne™ ABI, United States) by the gene-specific oligonucleotide primers (**Table 2**) synthesized commercially (Takara Biotechnology Co., Ltd, Dalian, China).

The real-time PCR was performed by SYBR Green I (Takara Biotechnology Co., Ltd). In this step, the PCR reagents included 12.5 µL of 2×SYBR Premix Ex Taq, 1 µL of each primer (5 mM), and 5µL of sample cDNA. The final volume was 25 µL. The PCR reactions were then performed as follows: 95 ºC for 2 min, [94 ºC for 30 s, 56 ºC for 20 s and 72 ºC for 30 s] (40 cycles). The melting curves after PCR were obtained using the stepwise increase of the temperature from 70-94 ºC.

*3.9. Analysis of Recombinant Albumin Production Using Densitometry*

SDS-PAGE, Bradford, and densitometry (Image J Software) methods were used to determine and quantify expressed recombinant (17). Densitometry is a quantitative method to measure the optical density (OD) in light-sensitive materials and can be used for proteins located on membranes or gels (18). SDS-PAGE on a polyacrylamide 12.5% (w/v) was performed for the determination of rh-Albumin expression level. The

**Table 2**. Oligonucleotide primers for real-time PCR.

| Gene | Primer | Primer sequence |
|---|---|---|
| **16s rRNA (internal control)** | *Forward* | 5'-AGAGTTTGATCCTGGCTCAG-3' |
| | *Reverse* | 5'-GGTTACCTTGTTACGACTT-3' |
| **ackA** | | |
| | *Forward* | 5'-TCACTGGTGGTATCGGTGAA-3' |
| | *Reverse* | 5'-ACCAGTTCTTCGTTGGTTGG-3' |

proteins were visualized using Coomassie brilliant blue R250 and quantified by gel densitometer. We used the Bradford protein assay to measure the concentration of total protein (19).

*3.10. Statistical Analysis*

Statistical analysis of data was calculated by SPSS software. Errors were determined as standard deviations, and differences between samples were tested by ANOVA. Statistical significance was considered at $P < 0.05$. The experiments were repeated at least three independent times.

## 4. Results

*4.1. Western Blot Analysis of Rh-Albumin*

The expressed rh-Albumin was analyzed using Western blotting by peroxidase-conjugated anti-His-tag monoclonal antibody. According to **Figure 1**, western blotting analysis confirmed the production of rh-Albumin in transformed bacteria.

*4.2. Preparation of ASO and PAMAM Complexes*

PAMAM was complexed with ASO in PBS. Positive/negative charge ratios for PAMAM/ASO complexes were 1/1, 1/2, 2/1, and 3/1. Using gel electrophoresis, it was determined that the ASO-dendrimer complexes were not electrophoresed and were retained in the sample. At the same time, the free ASOs were electrophoresed and migrated in the agarose gel. The complex with N/P ratios of 1:1, 2:1, and 3:1 showed complete complexation of ASO, but only partial complexation was found with the N/P ratio of 1:2 (**Fig. 2**). In the current study, 1:1 N/P ratio was selected to cut the costs.

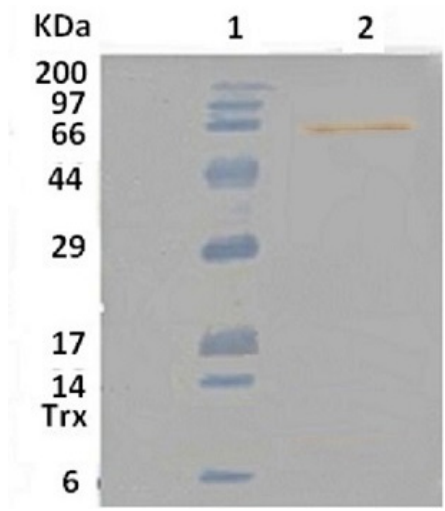


**Figure 1.** Western blot analysis of rh-Albumin expression. Lane 1: Molecular weight marker, lane 2: produced rh-Albumin.

*4.3. Cytotoxicity Assay*

Microdilution method showed MIC = 187.5 $\mu g.mL^{-1}$ for dendrimer, in concentration range of 5.85 to 1500 $\mu g.mL^{-1}$. Also, antisense didn't show any toxicity in the concentration range of 1.17 – 300 $\mu g.mL^{-1}$. 1:1 ratio of dendrimer/ASO complexes didn't show any cytotoxicity in the allowed concentration of dendrimer and ASO according to the above MIC reports.

*4.4. Expression Analysis of AckA in Antisense-Treated Bacterial Cells*

Following the treatment of *E. coli* with antisense, by quantitative real-time PCR assay, the expression level of ackA mRNA was determined. After 4 h of treatment, RNA was extracted and reverse transcribed to cDNA. As shown in **Figure 3**, the expression level of ackA

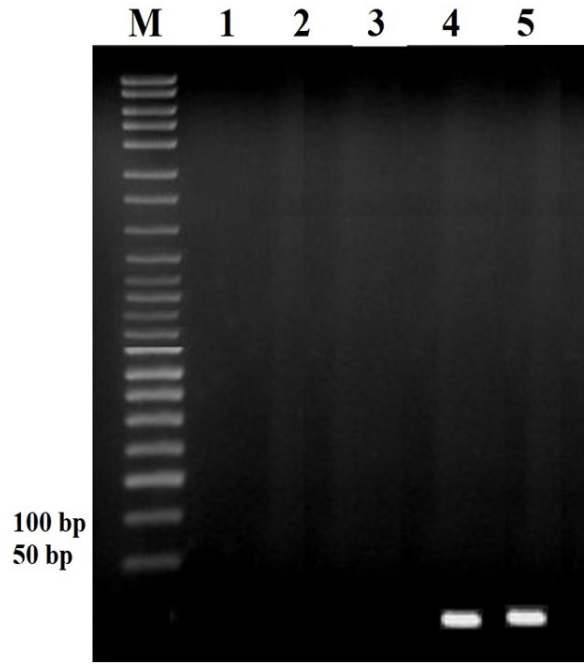


**Figure 2.** Agarose gel electrophoresis of ASO complexes with PAMAM dendrimers prepared with different N/P ratios. Lane M: molecular weight marker, Lane 1: 1;1 N/P ratio, Lane 2: 2;1 N/P ratio, Lane 3: 3;1 N/P ratio, Lane 4: 1;2 N/P ratio, Lane 5: ASO.

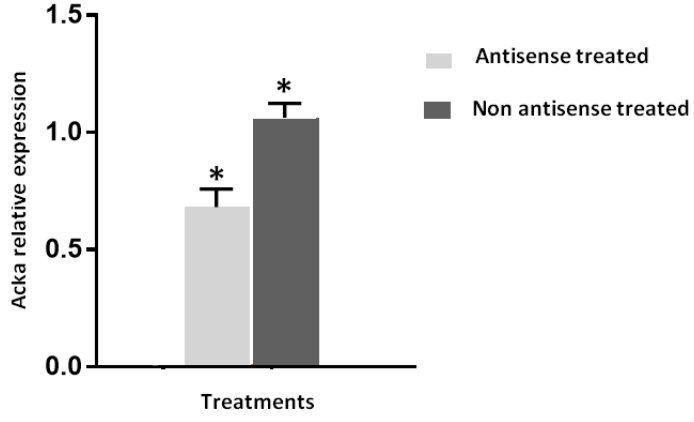


**Figure 3**. The expression level of ackA mRNA was down-regulated in the presence of antisense using real-time PCR assays. Analysis of data showed that antisense treatment could significantly decrease the relative expression of ackA mRNA compared with the non-treated group (P-value ≤ 0.05).

mRNA significantly decreased in antisense-treated cells relative to the controls ($p < 0.05$).

*4.5. Effect of Antisense Treatment on the Growth of E. coli BL21(DE3)*

As shown in **Figure 4**, treatment with antisense and decreasing acetate expression resulted in increased bacterial growth.

*4.6. Effect of Antisense Treatment on rh-Albumin Expression in Transformed E. coli*

In this study, after treating bacterial cells with antisense and decreasing the acetate concentration, we observed a 2.1 fold increase in recombinant albumin concentration compared to untreated cells (**Table 3**) (**Fig. 5**).

## 5. Discussion

Acetic acid (acetate) is an unfavorable extracellular

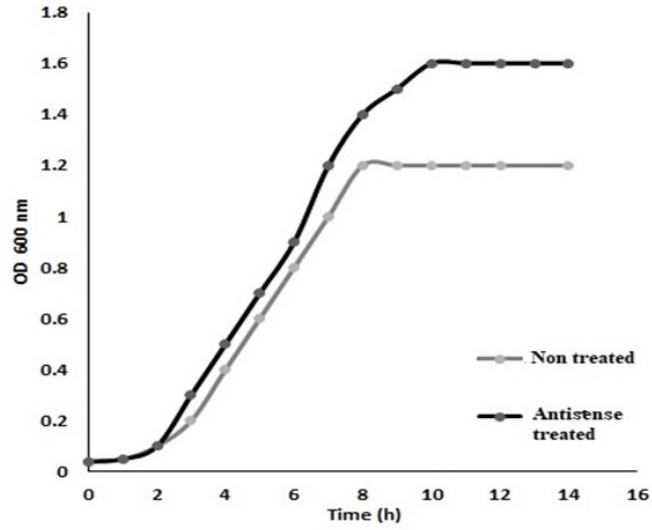


**Figure 4.** Effect of antisense treatment on growth kinetics of *E. coli* BL21(DE3).

**Table 3**. Effect of antisense treatment of *E. coli* BL21(DE3) on rh-Albumin expression.

| Treatment | Inducer concentration | Total protein (mg/ml) | Expression percent base of densitometery (%) | Alb concentration (mg/ml) | OD600 6h after induction | DCW (mg/ml) |
|---|---|---|---|---|---|---|
| **Antisense treated** | 0.5 mM | 3.35 | 37 | 1.25 | 1.5 | 5.45±0.04 |
| ***Non-antisense treated*** | *0.5 mM* | *2.39* | *25* | *0.59* | *1.2* | *4.27±0.02* |

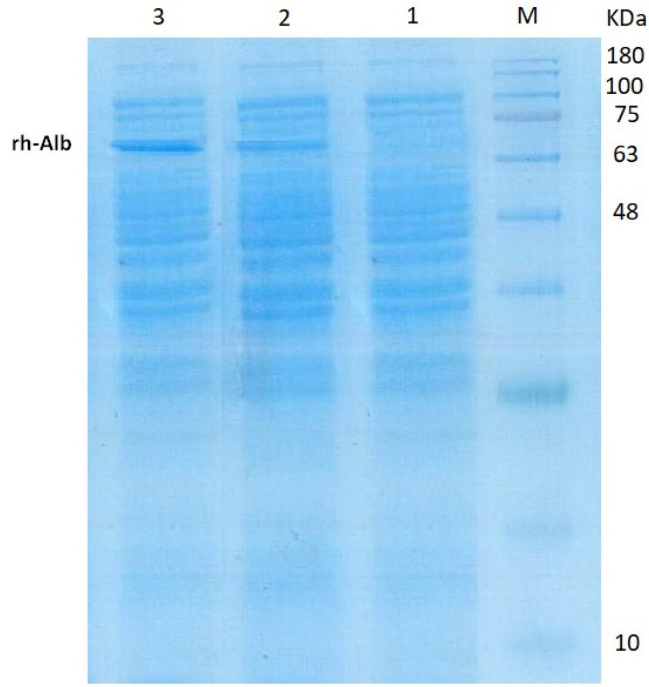


**Figure 5.** SDS-PAGE gel of rh-Albumin expression in transformed *E. coli*. Lane M: molecular weight marker, Lane1: before induction. Lane 2: 4 hours after induction without antisense treatment, Lane 3: 4 hours after induction in the presence of antisense.

by-product of *E. coli* with negative effects on protein production. High-density cell cultures of *E. coli* for recombinant protein production unavoidably lead to the accumulation of acetate and subsequently reduction of recombinant protein yield (20). Several strategies have been reported to reduce acetate accumulation and increase recombinant protein yield (21). These strategies include process improvement and modification (for example, optimization of cell culture condition and acetate elimination) and genetic modification of bacterial strains to decrease acetate synthesis (5, 22).

Antisense oligonucleotide (ASO)-based strategies provided an approach to reduce the expression of target mRNA (23).

Some studies have attempted to employ an antisense RNA approach to partially inhibit acetate kinase (ack) and phosphotransacetylase (pta) gene expression and bosynthesis (6, 22, 24). Kim and coworkers constructed three recombinant plasmids composed of antisense cassettes targeting either pta and ackA and used green fluorescent protein (GFP) as a target protein. They showed this approach decreased the expression of target genes and acetate accumulation in culture media. They also showed that the total GFP enhanced 1.6 to 2.1 fold in antisense-treated group (6). Tammala *et al*. used an antisense RNA against ctfB (the second CoAT gene on the polycistronic aad-ctfA-ctfB message) to increase the butanol/acetone ratio in Clostridium acetobutylicum ATCC 824 fermentation process (25).

Bakhtiari *et al*. inhibited ackA and pta gene expression through two specific antisense RNAs cloned sequentially in pBluescriptsk$^{+}$ and found reduced expression of the target genes, decreased the acetate production concentration in growth media and enhanced the growth rate of antisense-treated strain (26). In another work, Bakhtiari *et al*. down-regulated of ackA-pta pathway in *Escherichia coli* BL21 (DE3) using a recombinant plasmid containing specific antisense sequences for pta and ackA and showed this cassette could affect acetate production and also bacterial growth rate. (26). Wang *et al*. identified a new function of biotin carboxylase II encoded by yngH gene in *Bacillus subtilis* TS1726 via antisense RNA strategy. According to their results, this enzyme plays an important role in maintaining acetyl-CoA carboxylase (ACCase) activity and enhancing surfactin synthesis (27).

PAMAM dendrimers, due to their unique characteristics, have gained increasing attention as a delivery device for both plasmid DNA and antisense oligonucleotides transformation. (28). In this study, we examined the effect of acetate kinase A antisense on reduction of acetate expression in *E. coli also* used PAMAM dendrimers as a delivery agent. Our data showed that following the treatment of *E. coli* with antisense, the expression level of ackA mRNA was significantly decreased in antisense-treated group compare to the control group. Here, in order to study the effect of acetate reduction on the production of recombinant protein in bacteria, we used the recombinant plasmid (pET22b/HSA) expressing recombinant human albumin. After pET22b/HSA transformation into host cells and induction of recombinant E. coli BL21 by IPTG, the expression of recombinant albumin was analyzed. We found a significant increase in albumin expression in antisense-treated group. Results showed that antisense strategy could be a effective approach to enhance recombinant protein product rate.

## 6. Conclusion

Aerobic growth of *Escherichia coli* in medium contains glucose results in acetate production as the main by-product. At high concentrations, acetate bacterial growth and decreases cell mass and recombinant protein production. So, various strategies like medium composition optimization, acetate filtration, and gene suppression were taken to decrease acetate concentration in the culture medium. In this study, we designed an antisense oligonucleotide to suppress ack A expression as an important gene in the acetate production pathway in *E. coli.* PAMAM dendrimer was used as a suitable delivery agent because of some good features like uniformity, multivalency, and high biocompatibility. Real-Time PCR assay showed the level of ackA mRNA significantly decreased in antisense-treated group compare to the controls. Subsequently, in treated group cell mass (5.45 mg.mL$^{-1}$) and recombinant Albumin production (1.25 mg.mL$^{-1}$) significantly increased compare to the control group (4.27 mg.mL$^{-1}$ and 0.59 mg.mL$^{-1}$) respectively.

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