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Utilization of Cheese Whey for Production of Azurin by *Pseudomonas aeruginosa*

Yağmur ÜNVER[*1]

Abstract

Azurin which has attracted much attention as potential anticancer agent in recent years is a bacterial secondary metabolite. This copper-containing redox protein secreted by *Pseudomonas aeruginosa* has capability of preferentially entering into many human cancer cells and inducing apoptosis. In this study, whey which is the considerable by-product of the casein or cheese manufacture was used as azurin production medium by *P. aeruginosa*. Also, effects of copper (II) sulphate ($CuSO_4$) and potassium nitrate (KNO_3) on the azurin production were determined. At the end of the studies, optimum azurin expression level was reached during the incubation of 18 hours. The best $CuSO_4$ concentration was 2.5 mg/L while the best KNO_3 concentration was 45 mg/L according to Western blot analysis. This process can be used to obtain high levels of azurin using *P. aeruginosa* in whey medium. Also, using whey for azurin production can reduce many processing industrial whey waste management problems.

Keywords: azurin, *Pseudomonas aeruginosa*, secondary metabolite, expression, whey

1. INTRODUCTION

Natural products which are also referred to as specialized metabolites or secondary metabolites are produced by microorganisms and plants. These are a group of complex bioactive compounds which have unusual chemical properties and low-molecular weight structurally diverse [1, 2]. Antibiotics, growth hormones, pigments, antitumor agents, organic acids, nutraceuticals and others, are known as microbial secondary metabolites and not essential for the growth of microorganism. But, they have useful effects for human and animal health. Up to now, microbial secondary metabolites with their great potential have been used for other applications apart from antiviral, antibacterial and antifungal infections [3, 4].

Natural products and their derivatives are of great importance as they make up more than 40% of the therapeutic drugs such as antitumour, antibiotics and cholesterol-lowering agents [3, 5]. Cancers which are a great threat to humans are the second leading cause of death after cardiovascular disease [6]. Currently, a complete cancer remission often fails to achieve with the conventional cancer treatments such as chemotherapy, surgery and radiotherapy. Also, significant side effects have been recognized by radiotherapy and/or chemotherapy. Therefore, lots of new approaches for the treatment of cancer

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have been improved. Some of them, use of live, attenuated bacteria or their native purified [7] and recombinant products [8, 9]. Cancer targeted drugs, which provide to treat tumour and reduce side effects have shown rapid progress in recent year [7, 10, 11]. Azurin, one of them, is a redox protein containing copper and secreted by *Pseudomonas aeruginosa* which is a Gram-negative bacterium and can cause disease in certain sensible individuals [12]. Azurin has attracted much attention in the last two decades, because it preferentially enters into many human cancer cells and induce apoptosis [8,13–15]. This protein and its derived peptide p28 have anticancer activity that has been confirmed in mouse-based tumor models and various cancer cells [16, 17].

Whey which is the considerable by-product of the casein or cheese manufacture composes of 80% to 90% of the processed milk and contains about 55% of milk nutrients. Several factors such as milk quality, feed and animal breed are effect on whey composition and whey has about 6-10 g/L of proteins and a high lactose concentration (about 45 g/L) [18–20]. It is also rich in mineral salts (0.5-0.7 w/v), lipids (0.4-0.5 w/v) and minor components such as citric and lactic acids and B group vitamins, etc. [21]. Worldwide, around 190 $x10^6$ ton/year of whey production is estimated and it is known that about 9 liter of whey is produced in every 1 kg of cheese made [22, 23]. So, serious environmental problems arise due to high lactose content and discharging of whey in water systems without pre-treatments can not be possible [19]. Biotechnology, medical, agri-food and related industries exploit the whey because it is an ideal source of functional proteins and peptides, vitamins, lipids, lactose and minerals [24]. In this study, cheese whey was used as an alternative material for azurin production medium to defined media. Also, effects of copper (II) sulphate ($CuSO_4$) and potassium nitrate (KNO_3) on the azurin expression were determined. This is the first report on the production of azurin in whey medium.

2. MATERIAL and METHODS

2.1. Strain and Medium

P. aeruginosa ATCC9027 was obtained from Ataturk University, Department of Food Engineering, Microbiology Research Laboratory. Bacterial cells were incubated on LB (Luria-Bertani) agar plate to maintain the culture. Whey was obtained from a cheese plant in Erzurum. It was autoclaved at 121°C for 15 minutes and after being kept at +4 °C for a night, yellow whey was used as production medium. The production medium composed of whey (50 mL), 5 g/L $(NH_4)_2SO_4$, 2.5 g/L KH_2PO_4, 2,5 g/L Na_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $MnSO_4.7H_2O$, 5 mg/L $CuSO_4$ and 30 mg/L KNO_3. Initial pH value of the medium was adjusted to 6.5.

2.2. Azurin production in whey medium

Bacteria were streaked on LB agar and incubated for a night. Then, one colony was transferred into 10 mL LB medium and grown at 37 °C and 150 rpm during a night. After incubation, 1 mL of inoculum (OD_{600} ~1.6) was transferred to 50 mL production medium in a shaking flask. Then, it was closed by a cotton and an aluminum foil. To determine optimum azurin production time, culture medium was incubated at 37 °C and 150 rpm for 6 h, 12 h, 18 h and 24 h. At the end of each incubation times, culture liquids were collected and centrifuged at 9000 rpm and +4 °C for 10 minutes. After centrifugation, supernatants were discarded and cells were used for both preparing cell lysates and determination of wet cell weight as g/L.

2.3. Effect of copper (II) sulphate ($CuSO_4$) and potassium nitrate (KNO_3) on the production of azurin in whey medium

To obtain the highest azurin production, the influence of different concentrations of $CuSO_4$ (2.5 mg/L, 5 mg/L, 7.5 mg/L and 10 mg/L) and KNO_3 (15 mg/L, 30 mg/L, 45 mg/L, 60 mg/L and 75 mg/L) were studied at shaking-flask level, separately. Culture liquids were collected and centrifuged at 9000 rpm and +4 °C for 10 minutes.

After centrifugation, supernatants were discarded and cells were used for both preparing cell lysates and determination of wet cell weight as g/L.

2.4. Extraction of cellular protein

Extraction of proteins from bacterial cells was modified from Ramachandran et al. (2012). After cultivations, 2 ml of culture liquid obtained from each culture was centrifuged as described above. Cell pellets were washed with sterile distilled water and centrifuged again. Then, the cells were suspended in 500 µL, 0.02 M potassium phosphate buffer (pH 7) containing 1mM PMSF (phenylmethylsulfonyl fluoride) as protease inhibitor. The cell suspensions were sonicated by keeping in the ice basket using eighteen 10 second bursts at high intensity and a 10 second cooling period between each burst was performed. Then, sonicated suspensions were stirred vigorously and centrifuged at 9000 rpm and +4 °C for 10 minutes. After centrifugation, the supernatants were transferred to fresh tubes and stored on ice [25].

2.5. Western blot analysis

To determine the optimal values of incubation time, concentration of copper sulphate and potassium nitrate for azurin expression, Western blot analysis was used. For this purpose, obtained cell lysates were used and total protein amounts of the lysates were determined by Bradford method [26]. Then, the cell lysates which included equal amount of total protein (for determination of optimum incubation time, $CuSO_4$ and KNO_3 concentrations, total protein amount was used as 60 µg, 50 µg and 40 µg, respectively) and 2X SDS-PAGE sample buffer were mixed together and boiled for 5 minutes to run on SDS-PAGE gel. After gel electrophoresis, proteins were blotted to a PVDF (polyvinylidene difluoride) membrane from the gel by transfer at 25 V and 2.5 A for 7 min. PBST (Phosphate Buffered Saline with Tween 20; 1.8 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl and 0.05% Tween 20) containing 5% skim milk powder were used for blocking the membrane for 1h. The membrane was incubated with anti-azurin antibody (Sicgen) for overnight after it was washed with PBST three times. At the end of the incubation, the membrane was incubated with peroxidase conjugated rabbit anti-goat immunoglobulin G (Anti-Goat IgG H&L (HRP), Abcam) as the secondary antibody for 1 h, after it was washed with PBST six times. Then, the membrane was washed with PBST. Supersignal® West Femto and Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) were used for visualization of the protein bands by ChemiDoc™ Touch Imaging System (Bio-Rad). Based on this analysis results, the relative expression levels of azurin were quantified with ImageJ software and compared to each other. Tukey Test (One-way ANOVA) was used for statistical analysis and $P<0.05$ was regarded as significant using GraphPad Prism 5.

3. RESULTS and DISCUSSION

3.1. Determination of incubation time for optimum azurin production

Yamada *et al*. (2002) reported that azurin enters into the cytosol of the human melanoma cell line (UISO-Mel-2), transports to the nucleus and stabilizes the protein by forming a complex with p53 [27]. Punj *et al*. (2004) also found that azurin shows strong cytotoxic activity in MCF-7 breast cancer cell line and increases apoptosis density by more than 50%, while reported that it increases apoptosis density by 15-18 %, in MDA-MB-231 and MDA-MB-157 cells [28]. Similarly, Choi *et al*. (2011) observed a decrease in the viability of oral squamous cancer cells treated with azurin, DNA breakage, morphological changes and an increase in cyclin B1 and p53 protein levels [29]. Therefore, novel methods have been searching by researchers to enhance the production of azurin which has been known to be a potential anticancer protein [30]. In this regard, this study aimed to enhance the expression of azurin in *P. aeruginosa* in low-cost whey medium. Since the existence of a copper ion in the polypeptide chain of azurin is known to contribute to stability of azurin [31], copper sulphate was also added to the culture medium. On the other hand, a comparatively high concentration of $(NH_4)_2SO_4$ (5 g/L) was added to the medium so that KNO_3 was used only for denitrification and not as a source of nitrogen

[32]. To determine optimum incubation time for azurin production in whey medium, bacterial cells were incubated during 24 hours. 2 ml of culture liquids were taken in 6th, 12th, 18th and 24th hours. Because azurin protein with the secretion signal peptide is located in the periplasm [33], the cells were lysed by sonication and obtained lysates were analysed by Western blotting. According to this analysis result, azurin was observed in expected size (14 kDa). Maximum azurin expression was obtained in 18th hour and a decrease of azurin expression was observed after this time. On the other hand, it was shown that cells had the lowest azurin expression in 6th hour (Figure 1a and 1b). Because azurin is a secondary metabolite, it is produced by *P. aeruginosa* in stationary phase of growth. Similarly, Vijgenboom *et al.* (1997) reported that there was an increase in azurin expression when shifting from exponential to stationary phase. Also, as described these researchers, the upper band of azurin in the lanes may not associated with azurin and is a cross-reaction of the anti-azurin antibody [34]. On the other hand, an increase in cell biomass was observed up to the 18th hour while no significant difference was observed between cell weights belong to 18th and 24th hours (Figure 1 c).

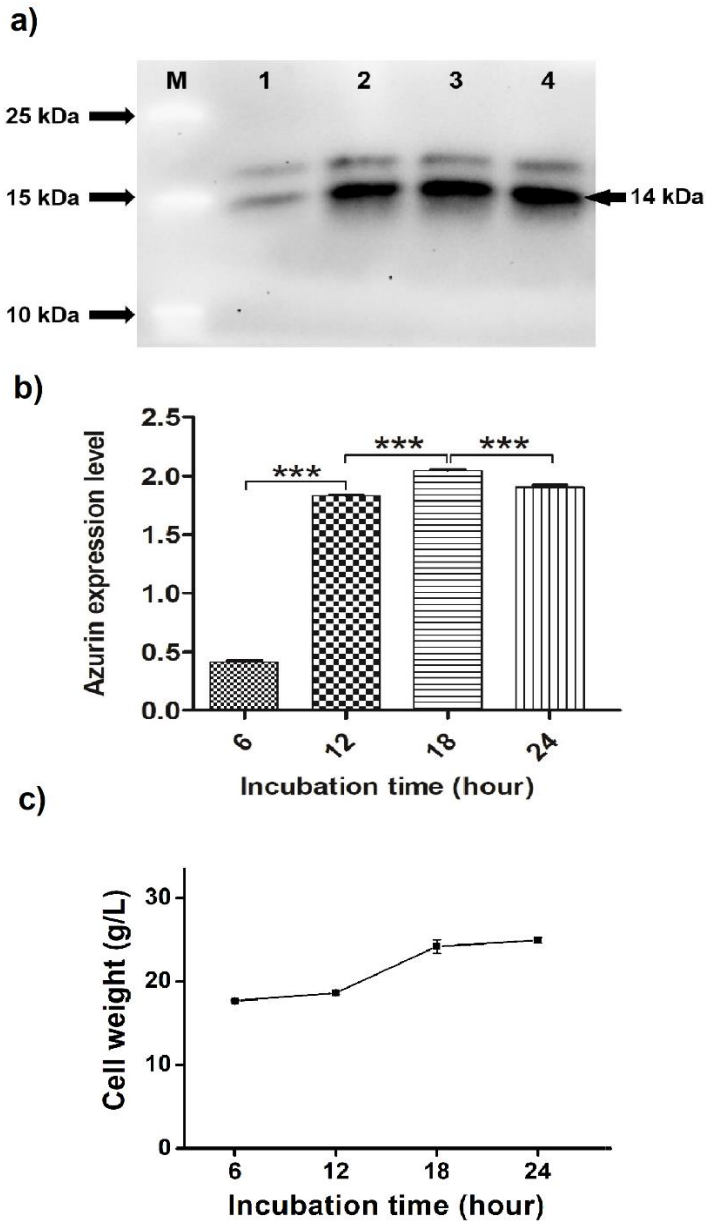

Figure 1 Effect of incubation time on expression level of azurin and cell growth, a) Western blot analysis result of samples from cell lysates (M: Marker, 1: 6 h, 2:12 h, 3:18 h and 4:24 h) b) The relative expression levels of azurin, c) Wet cell weight for incubation time.

3.2. Effect of copper (II) sulphate ($CuSO_4$) on azurin production in whey medium

Sutherland (1966) reported that the copper content of the culture medium affect azurin content of the cells and azurin was nearly absent in the cells when the copper content was lower

than 0.5 mg/L in the medium. On the other hand, it was reported that while the azurin content of cells grown in a medium containing 0.5-5 mg/L copper increased, the copper content above this range did not lead to increase of azurin content [35]. Therefore, in this study, different $CuSO_4$ concentrations (2.5 mg/L, 5 mg/L, 7.5 mg/L and 10 mg/L) were added to the culture media to determine the optimum $CuSO_4$ concentration used as the source of copper ions. After 18 h incubation, obtained cell lysates were analyzed by Western blotting. According to the results (Figure 2a and 2b), 2.5 mg/L $CuSO_4$ was lead to maximum azurin expression. Namely, presence of copper ion in whey medium was lead to enhance azurin synthesis. Over this concentration, as concentration of $CuSO_4$ increase, a decrease of expression level of the protein was observed. Similarly, Ramachandran *et al*. (2012) reported that adding copper in the culture medium was lead to both enhance azurin synthesis and reveal the differences of secondary structure stability of azurin expressed in *P. aeruginosa* [25].

3.3. Effect of copper sulphate on cell growth in whey medium

After 18h incubation, cells were harvested from culture media which contain different concentrations of $CuSO_4$ and wet cell weight of the cells were calculated. According to the results, a decrease in biomass yield was observed over 7.5 mg/L $CuSO_4$ concentration (Figure 2c). This situation might be attributed to toxicity of copper ions to *P. aeruginosa* cells over this concentration.

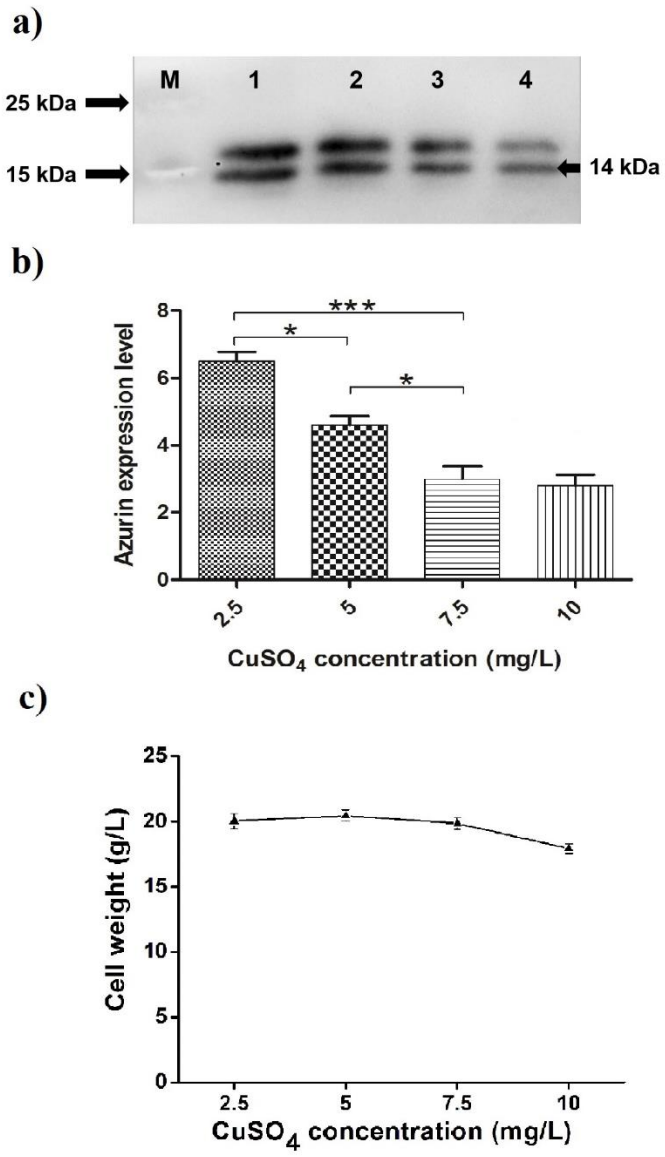

Figure 2 Effect of $CuSO_4$ concentration on expression level of azurin and cell growth, a) Western blot analysis result of samples from cell lysates (M: Marker, 1: 2.5 mg/L, 2: 5 mg/L, 3: 7.5 mg/L and 4: 10 mg/L), b) The relative expression levels of azurin, c) Wet cell weight for different $CuSO_4$ concentrations.

3.4. Effect of KNO_3 on azurin production in whey medium

P. aeruginosa can perform denitrification in the presence of nitrate and under anaerobic conditions. In this respiratory process, nitrate is reduced to nitrogen gas via nitrite (NO_2), nitric oxide (NO) and nitrous oxide (N_2O). In this way, produced nitric oxide by nitrite reductase causes

intracellular damage, whether nitric oxide reductase does not immediately convert nitric oxide to nitrous oxide. To avoid this damage, a stress response can be induced with a higher expression of azurin as potential electron donor [31]. So, in this study, the optimum KNO_3 concentration used only for denitrification was determined to achieve a high level of azurin expression in whey medium. For this purpose, different concentrations of KNO_3 (15 mg/L, 30 mg/L, 45 mg/L, 60 mg/L and 75 mg/L) were added to the culture media. After 18 h incubation, obtained cell lysates were analyzed by Western blotting. According to the results, expression level of azurin was increased related to increasing of KNO_3 and 45 mg/L KNO_3 was lead to maximum azurin expression (Figure 3a and 3b). When using KNO_3 concentrations of 60 mg/L and 75 mg/L above this concentration, a significant decrease in the level of protein expression was observed. This situation might be related to increasing nitric oxide level in the cells. In the respiratory process of these cells high level of nitrate in the medium caused high level of nitric oxide (NO) by reduced and nitric oxide reductase amount in the cells was not enough for immediately convertion of all nitric oxide to nitrous oxide. Therefore, increased nitric oxide level in the cells induced intracellular damage that caused decreasing of azurin expression level.

3.5. Effect of KNO_3 on cell growth in whey medium

After 18h incubation, cells were harvested from culture media which contain different concentrations of KNO_3 and wet cell weight of the cells were calculated. According to the results, biomass yield was increased related to increasing of KNO_3 and maximum wet cell weight was observed in the concentration of 45 mg/L KNO_3. Above this concentration, a decrease in the biomass yield was observed. This might be related to increasing nitric oxide level induced intracellular damage in the cells as seen in the decreasing level of azurin expression after the same KNO_3 concentration. An occured intracellular damage might cause a decrease of cell biomass (Figure 3c).

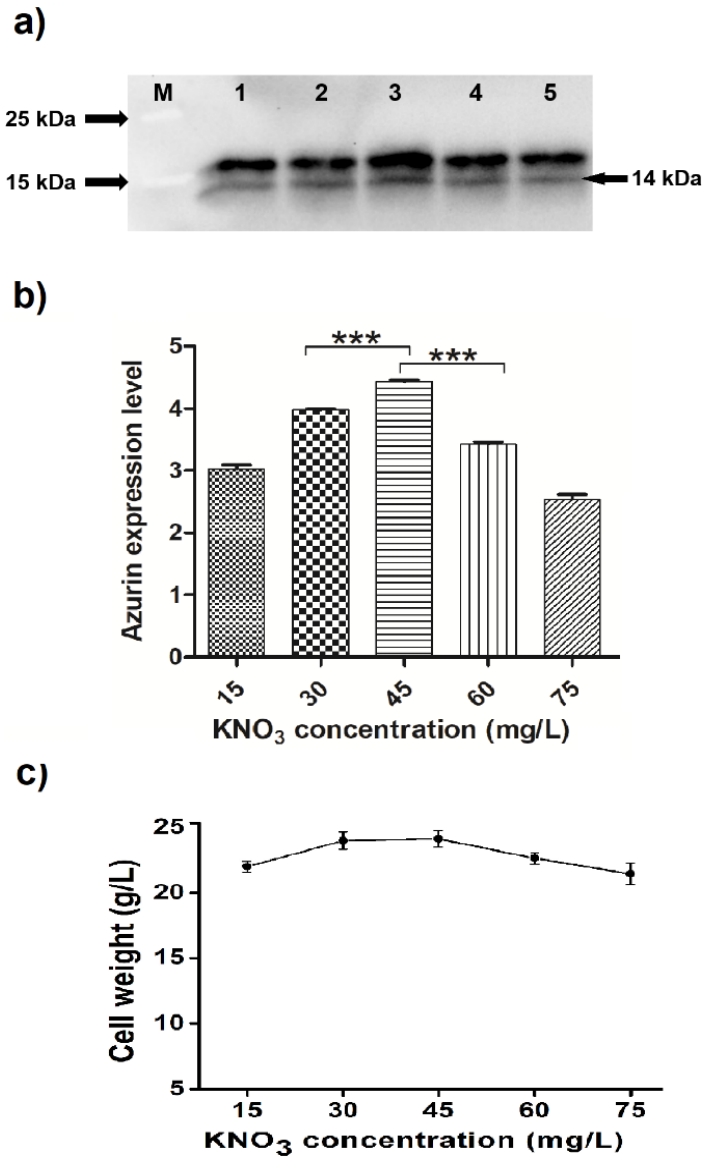

Figure 3 Effect of KNO_3 concentration on expression level of azurin and cell growth, a) Western blot analysis result of samples from cell lysates (M: Marker, 1: 15 mg/L, 2: 30 mg/L, 3: 45 mg/L, 4: 60 mg/L and 5: 75 mg/L), b) The relative expression levels of azurin, c) Wet cell weight for different KNO_3 concentrations.

4. CONCLUSION

The secondary metabolites are used for development of new chemotherapeutics and drugs. Azurin, which is a bacteriocin, has a cytostatic property with its penetration to breast cancer cells. So, it can be used as a potential anticancer agent. In this study, the production of the azurin protein was carried out for the first time in whey medium by *Pseudomonas aeruginosa*. Furthermore, the effective expression of this therapeutic protein was obtained with the addition of $CuSO_4$ and KNO_3 in whey medium. So, the high-level azurin production was achieved in a cost-effective medium and also, using whey for azurin production can reduce many processing industrial whey waste management problems.

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the author.

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