ارزیابی تولید پروتئاز *آئرومونـاس هیدروفیلا* MSB16 در پاسخ به جریان الکتریسیته با شدت کم، سورفاکتانت ها و نانوذرات

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چکیده

مقدمه: تولید مقرون‌به‌صرفهٔ پروتئازها یکی از چالش‌ها در صنعت آنزیم است و گزارشی در زمینهٔ تأثیر جریان الکتریسیته با شدت کم و نانوذرات بر تولید پروتئازهای خنثی موجود نیست. هدف مطالعهٔ حاضر، افزایش بازده تولید پروتئاز از *آئرومونـاس هیدروفیلا* MSB16 با استفاده از این تیمارهاست.

مواد و روش‌ها: در مطالعهٔ حاضر، نانوذرات مختلف مانند نقره، آهن سه‌ظرفیتی، آهن، آلومینیوم، روی و مس در غلظت 2ppm و جریان الکتریسیته با شدت 50µA استفاده شد؛ به‌علاوه، تولید پروتئاز MS16 در حضورSDS، Tween 80، Triton X-100، Span 80 و گلیسرول بررسی شد.

نتایج: قرارگرفتن سلول‌های باکتریایی در فاز سکون رشد در معرض جریان الکتریسیته به‌مدت ۱۰ و ۲۰ دقیقه تولید پروتئاز در MSB16 را به‌ترتیب ۴۸/۲ و ۵۹/۱ درصد افزایش داد؛ همچنین، گلیسرول، Tween 80، Span 80 و نانوذرهٔ نقره به افزایش تولید پروتئاز به‌ترتیب برابر با ۵۶/۴، ۴۰/۴، ۲۴/۸ و ۱۲/۵ درصد منجر شدند.

بحث و نتیجه‌گیری: درمجموع، سورفاکتانت‌های غیریونی، گلیسرول و جریان الکتریسیته سبب افزایش تولید پروتئاز سویهٔ MSB16 شدند. تأثیر جریان الکتریسیته برتولید پروتئاز سویهٔ MSB16 به فاز رشد باکتری بستگی دارد.

واژه‌های کلیدی: *آئرومونـاس هیدروفیلا*، جریان الکتریسیته، گلیسرول، پروتئاز خنثی، بهینه‌سازی، توئین ۸۰

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Evaluation of *Aeromonas Hydrophila* MSB16 Protease Production in Response to Low-Intensity Electric Current, Surfactants, and Nanoparticles

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Abstract

Introduction: Cost-effective production of proteases is one of the challenges facing the enzyme industry. In this regard, there are no reports concerning the influence of low-intensity electric current and nanoparticles on microbial neutral proteases production. This study aimed at investigating the effect of these treatments on the neutral protease production of *Aeromonas hydrophila* MSB16.

Materials and methods: The protease production of *A. hydrophila* MSB16 in the presence of ionic and non-ionic surfactants (SDS, Tween 80, Triton X-100, and Span 80), glycerol as well as various nanoparticles (Ag, Fe III, Fe (0), Al, Zn, and Cu), was investigated. Furthermore, the effect of an electric current (50 µA) exposure to the bacterial cells during the logarithmic and stationary phase for 10 min and 20 min was studied.

Results: According to the results, electric current exposure to the bacterial cells entered the stationary phase for 10 min and 20 min increased the protease production of *A. hydrophila* MSB16 by 48.2% and 59.1%, respectively. However, this positive effect was not observed for log phase-bacterial cells. Besides, glycerol, Tween 80, Span 80 and Ag nanoparticles enhanced the protease production of *A. hydrophila* MSB16 by 56.4%, 40.4%, 24.8%, and 12.5%, respectively.

Discussion and conclusion: Non-ionic surfactants, glycerol, and electric current increased the protease production of *A. hydrophila* MSB16. Interestingly, the influence of electric current on the protease production of *A. hydrophila* MSB16 was dependent on the growth phase of the bacterium.

Key words: *Aeromonas hydrophila*; Electric Current; Glycerol; Neutral Protease; Optimization; Tween 80

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Introduction

The neutral proteases are an important group of commercial enzymes (1). For industrial usage, a commercial enzyme must perform significant activity and provide operational stability. Another challenge facing the enzyme industry is the affordable production of the enzymes, which motivated the researchers to find good protease producer strains and use different production media to increase the enzyme yield (2). Even a small improvement in protease production is critical and noticeable (3). Carbon and nitrogen sources, pH of fermentation medium, the temperature of incubation, and speed of aeration are among the most commonly studied factors in the previous studies (4-6). However, novel and cost-effective treatments that can increase the production of proteases are promising, because one of the challenges facing the enzyme industry is affordable enzyme production.

Previous studies have reported the positive effect of glycerol, nonionic or anionic surfactants on the production of lipase, cellulase, amylase, phytase, and lignase, but there are few reports concerning the effect of these compounds on the production and release of bacterial neutral proteases (7, 8). Besides, the use of surfactant resistant enzymes is an advantage in the industry (9). Therefore, using surfactants in the enzyme production medium has two advantages. First, it has a positive effect on the production and release of proteases into fermentative medium, and second it can help in the selection of surfactant-resistant enzymes. Surfactants or surface-active agents are amphiphilic molecules and they are classified based on their polar head as non-ionic, anionic or cationic, and zwitterionic or amphoteric surfactants (10). The most commonly used surfactant types in the industry are anionic and nonionic surfactants that are used in the detergent and food industry, respectively (11). However, cationic and zwitterionic surfactants are more expensive to produce and so they are for special use (12).

In addition, there are no previous reports regarding the effect of electric current and nanoparticles (NPs) on the production of neutral proteases. Nanoparticles,` (i.e., materials that have at least one dimension of 100 nm or smaller) have different physical and chemical interactions with biomolecules and cells due to their unique size concerning the same materials with larger size (13). They mostly are used for immobilization of microbial cells and their enzymes to increase biocatalyst stability, batch or continuous use and also to make separating the biocatalyst easier from the reaction mixture (14). There are conflicting reports about the effect of immobilization on the enzyme activity, however, some studies reported that immobilization of enzymes on NPs could enhance the enzyme activity (15). Furthermore, NPs cause physical destruction of the bacterial cell wall, so it is not irrational that they can increase the release of proteases to fermentative medium (16).

There are many reports concerning the effect of high-intensity electric current on microorganisms as an antibacterial treatment, but the effect of low-intensity electric current on microbial enzyme production is a less explored topic (17, 18). The low-intensity electric current had no antibacterial effect, however, it can influence the bacterial cell wall and increase the enzyme release (19). In addition, enzymes are more resistant to electric current concerning microorganisms and they are not adversely affected by electric current (18). Therefore, using low-intensity electric current can be a cost-effective treatment for increasing enzyme production. Based on the discussion above, the influence of ionic or non-ionic

surfactants, glycerol, nanoparticles (NPs), and low-intensity electric current on *Aeromonas hydrophila* MSB16 protease production was investigated in the present study to introduce novel and affordable treatments for the production of neutral proteases.

Material and Methods

Microbial Strain: *Aeromonas hydrophila* MSB16 (accession number KT006757) was previously isolated from the wastewater of a sausage factory (Isfahan, Iran) using skimmed milk agar (pH 7) (20). The primary identification of the strain based on the morphological and biochemical features was confirmed by almost full length (1419bp)16S rRNA gene sequencing using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′). The PCR mixture containing 2.5 µl PCR buffer, 0.5µl dNTP (10mM), 1µl $MgCl_2$ (50mM), 0.5µl each primer, 3µl template DNA, 0.2 µl Taq DNA polymerase (CinnaGen, Iran) and 17 µl dH2O in a final volume of 25 µl was used. The PCR reaction condition was 95°C for 5 min, followed by 30 cycles of 94°C for 30s, 54°C for 45s and 72°C for 1.5 min, with a final 5 min extension at 72°C (20). The phylogenetic analysis of the strain was performed using the software package MEGA, version 10 (21). Muscle program was used for the multiple alignments of data (Fig. 1).

Protease Production: *A. hydrophila* MSB16 was grown in the initial protease production medium (pH 7) containing only skimmed milk at the concentration of 20 g l^{-1} (30 ºC, 48 h, 180 rpm). The inoculum was prepared by adding a loop full of pure culture into 5 ml of sterile pre-culture medium, that is, tryptic soy broth (TSB). This inoculum was added to the initial culture medium at the concentration of 1% v/v (20). After 48 h of incubation, the medium was centrifuged at 4000 rpm (10 min, at 4 ºC) to obtain the cell-free supernatant for the determination of the extracellular protease production (1).

Protease Activity Assay: Enzyme production assay was carried out using the reaction mixture containing 0.5 ml cell-free supernatant and 2.5 ml casein solution (0.65% w/v) dissolved in phosphate-buffered saline (pH 7, 10 min, 37 ºC). Then, 2.5 ml trichloroacetic acid (10% w/v) was added to terminate the reaction. The released tyrosine content was evaluated in 1 ml supernatant after centrifugation at 10,000 rpm for 10 min using 2.5 ml sodium carbonate (0.5 M) and 0.5 ml Folin–Ciocalteau reagent (10% v/v). The absorbance of the resulted blue mixture was measured at 660 nm after 20 min incubation at 37 ºC. The amount of enzyme that could release 1µg of tyrosine per min per ml under the assay conditions (pH 7, 10 min, 37 ºC) was considered as one enzyme unit (22). All experiments were carried out in triplicate and the reported results were the averaged values.

Effect of Additives on Enzyme Production: Ionic and non-ionic surfactants such as SDS, Tween 80, Triton X-100 and Span 80 and also glycerol at the concentration of 0.1% v/v were added separately to the production medium (7, 8). In addition, NPs such as Ag, Fe III, Fe (0), Al, Zn and Cu at the concentration of 2ppm were added separately to the production medium to investigate the influence of NPs on the enzyme production. Due to the antibacterial effect of NPs, the addition of NPs to the fermentation medium was carried out after 24 h of incubation.

Effect of Electric Current on Enzyme Production: Electric current treatment was performed using an electrophoresis system (Padideh Nogen Pars, HU150, Iran) connected to a DC power supply (50 µA; 0.1 V; 3.4 mA m^{-2}) that was kindly

provided by the Biology Department of the University of Isfahan. The electrodes were 18 cm apart (platinum, 0.5 mm × 20 cm). Exposure to electric current was carried out after 4 h and 48 h of incubation to study its influence on enzyme production of the growing and stationary phase bacterial cells, respectively (23).

Statistical Analysis: Statistical analysis of the present study such as Duncan's multiple range tests and analysis of variance (ANOVA) was carried out using SPSS software (SPSS, Inc., Chicago, IL, USA). The statistical significance of the results was reported based on a p-value of less than 0.05.

Results

The phylogenetic analysis of the 16s rRNA sequence of *A. hydrophila* MSB16 is illustrated in Fig.1.

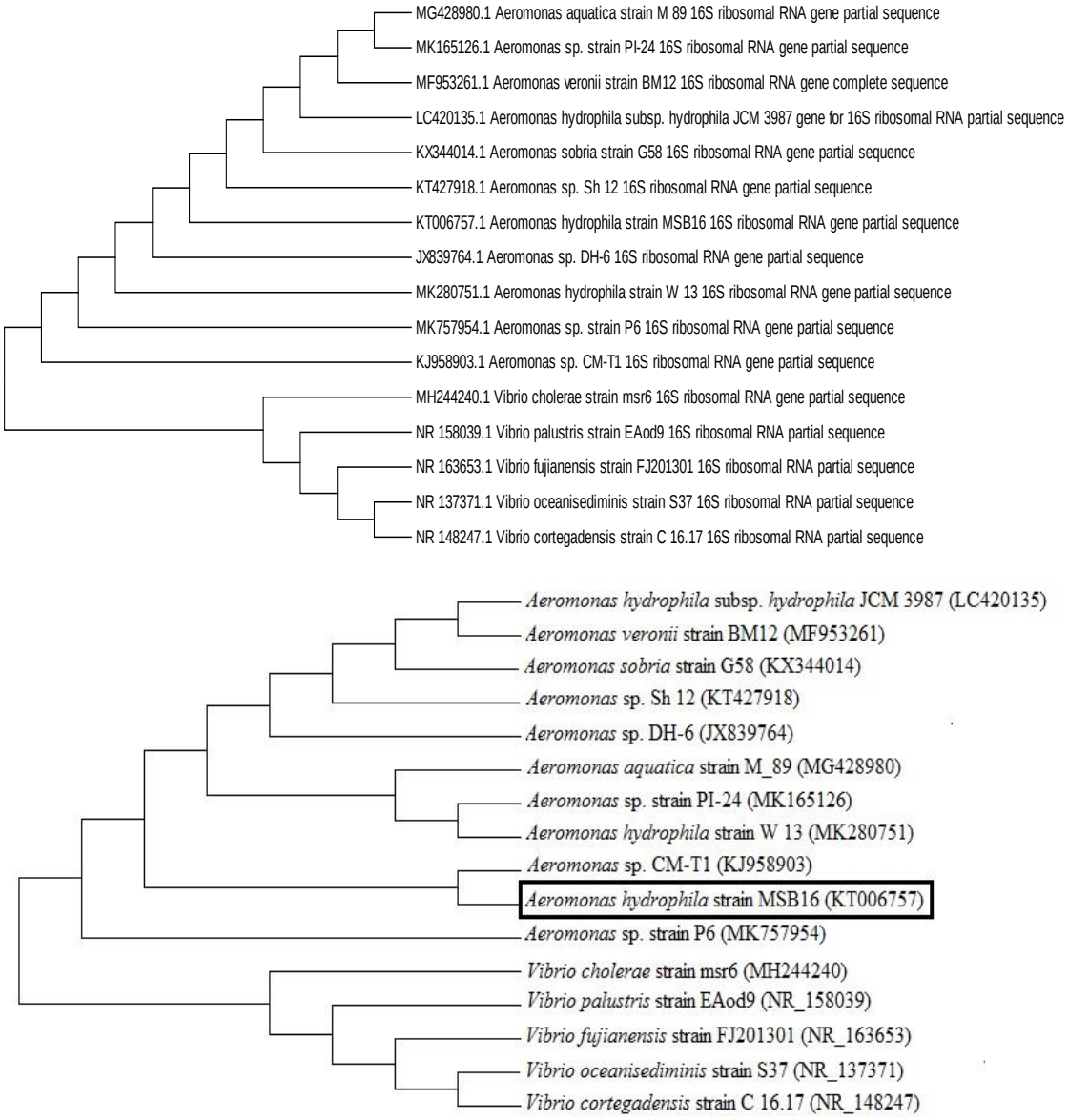

Fig. 1- Phylogenetic analysis of *Aeromonas hydrophila* MSB16, based on the 16S rRNA sequence comparison, using the maximum likelihood method. The numbers in the brackets are the accession numbers of the reference strains.

The results of electric current treatment showed that *A. hydrophila* MSB16 enzyme production increased by 48.2% and 59.1%, after exposure for 10 minutes and 20 minutes, respectively. However, this positive effect was seen only for the stationary phase cells but not for the growing cells.

The production of *A. hydrophila* MSB16 protease enzyme in the presence of the studied additives is illustrated in Figures 2 and 3. Glycerol as a polyol compound enhanced the enzyme production by 56.4%. Glycerol was the most effective additive in the production of *A. hydrophila* MSB16 protease.

Non-ionic surfactants in the present study, that is, Tween 80 and Span 80, but not Triton X-100 significantly increased the production of the protease enzyme. However, Tween 80 was the most effective additive among the studied non-ionic surfactants. In contrast, Triton X-100 did not have much positive effect on the enzyme production and its presence in the fermentation medium did not differ statistically significant concerning the control. On the other hand, the studied anionic surfactant, SDS, decreased *A. hydrophila* MSB16 enzyme production by 3% concerning the production medium without any additive (control).

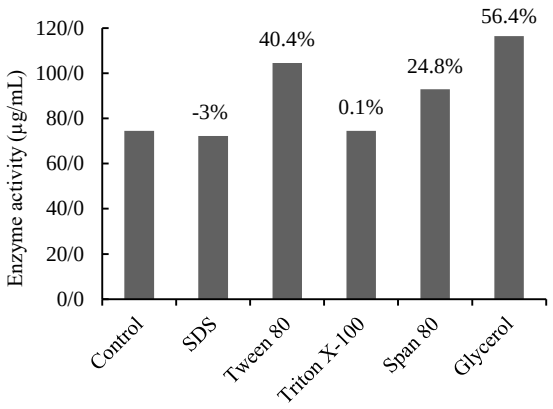

Fig. 2- Effect of surfactants and glycerol on the protease production of *A. hydrophila* MSB16. The results represent the means of three experiments. The numbers above each column indicate the percentage of increase (+) or decrease (-) in enzyme production with respect to the control.

Among the studied NPs, silver NPs had a significant influence. Nanoparticles of Cu, Fe (0) and Fe III had similar positive effects; however, zinc NPs had the minimum positive effect on *A. hydrophila* MSB16 enzyme production (Fig. 3).

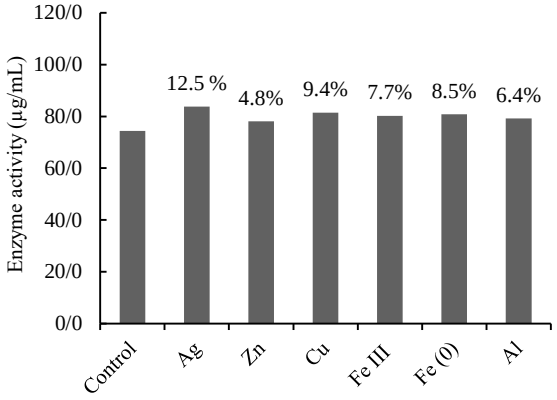

Fig. 3- Effect of nanoparticles on the protease production of *A. hydrophila* MSB16. The results represent the means of three experiments. The numbers above each column indicate the percentage of increase in enzyme production with respect to the control.

Discussion and Conclusions

In the present study, the influence of nanoparticles (NPs), and low-intensity electric current on a neutral protease production was investigated as affordable treatments for the first time. In addition, the effect of SDS, glycerol and some of the non-ionic surfactants such as Tween 80, Triton X-100, and Span 80 was also studied.

The results of the present study revealed that the phase growth of microbial cells could influence the effect of low-intensity electric current on enzyme production significantly. The reason can be explained as follows. The damage of the cell membrane caused by the electric current leads to the growing bacterial cell death which is the probable reason for this negative effect (17). However, the electric current treatment of bacterial cells after 48 h of incubation, which is the optimum time of producing the enzyme, leads to the release of more enzymes or the stimulation of

bacterial metabolism in favor of the enzyme production (19).

Among the studied additives in the fermentation medium of *A. hydrophila* MSB16, glycerol had the most positive effect. The reason is that glycerol is an enzyme or a protein stabilizing agent as well as the broth medium homogenizer (24). The effectiveness of glycerol on the enzyme production also has been shown in another study (25).

Non-ionic surfactants, especially Tween-80, also had a positive influence on the protease production of *A. hydrophila* MSB16 similar to some of the previous studies. For example, Evans and Abdullahi (2012), found a significant 5-fold increase in the production of *Bacillus subtilis* protease enzyme by Tween 80 and a significant increase in the thermal stability of the enzyme from 50 °C to 60 °C in the presence of this substance. Interestingly, it has been reported in their study that Tween 80 resulted in a 2.5-fold increase in cell growth and also a reduction in the lag phase of the growth curve (26). The presence of Tween 80 or polysorbate 80 in the broth medium is believed to increase its homogeneity, so the uptake of nutrients and oxygen increased by the microbial strains. This can explain why Tween 80 increased the growth rate and reduced the lag phase of the strain. Furthermore, Evans and Abdullahi (2012) observed that the enzyme properties like K_M, V_{Max}, the optimum pH and temperature of the enzyme activity did not change in the presence of Tween 80. Therefore, keeping the enzymatic properties as well as increasing the production and thermal stability of the enzymes are the advantages of using Tween 80 which makes it useful for enzyme production at the industrial level (26). In addition, Ananthan (2014) studied the effects of surfactants such as SDS, Tweens (20, 40, 60 and 80), PEG, and Triton X-100 at the concentration of 0.1% on the protease produced by *Vibrio* GA CAS2, and observed that only Tween 80 increased the production of enzyme, and other surfactants resulted in reduced enzyme production (27).

The physiology of the microorganism and/or the structure of the enzyme may subject to impair with increasing surfactant concentration. Therefore, at the higher concentration of surfactants in the fermentation medium, it is possible that the yield of enzyme synthesis decreases (28).

Therefore, the type of surfactant (ionic and non-ionic), its concentration and type of microbial strain are among effective factors to be considered in the studies on the influence of surfactants on enzyme production (7, 8, 29). The length of the hydrophobic chain of surfactants has also been reported to be effective in the enzyme production because surfactants that have a long chain can create a permeable cell membrane. As a result, it increases the uptake of nutrients into the cell or releases the enzymes from the cell (28).

In contrast to the non-ionic surfactants, the ionic surfactant (SDS) suppressed *A. hydrophila* MSB16 protease production. SDS is commonly used for lysing cells during RNA/DNA extraction due to the dissolution of the plasma membrane. Therefore, it seems to be effective for more release of the enzyme from the cell (30). But, SDS has been also used for denaturing proteins in the SDS-PAGE electrophoresis (31). Therefore, the reason for the decreased protease production by *A. hydrophila* MSB16 in the presence of SDS may be due to the denaturing of the enzyme. Besides, the negative effect of the enzyme production in the presence of SDS can be attributed to a higher rate of cell death in the presence of this substance. Anionic surfactants can bind to proteins, starch, DNA, and phospholipid membranes, which lead to loss of function of these components and ultimately the cell death and reduced the production of their metabolites (32).

Nanoparticles had a stronger positive effect on *A. hydrophila* MSB16 enzyme production than SDS and Triton X-100, but they had a less significant positive effect on the enzyme synthesis concerning the electric current and other studied non-ionic surfactants. The molecular mechanism of silver nanoparticle effect is related to the binding of these nanoparticles to the microbial cytoplasmic membrane and cell wall, resulting in their structural changes and finally pore formation in them. Through these pores, cellular compounds including the enzymes can be released out of the cell (33, 34). However, because of the interaction of these particles with phosphate compounds such as DNA and RNA, along with the antibacterial activity of these NPs, the optimum concentration of NPs must be determined. Furthermore, it was reported previously that the growth phase of bacteria can affect the influence of NPs on the production of the enzyme (23). In this study, all NPs were added to the fermentation medium before the inoculation of bacterial cells decreased the enzyme production (data not shown). Therefore, the NPs were added to the fermentation medium after 24 h of incubation when the number of bacterial cells in the culture medium and the production of the protease have increased.

Overall, non-ionic surfactants, glycerol, and electric current could increase *A. hydrophila* MSB16 protease production. However, the influence of electric current and nanoparticles was dependent on the growth phase of bacterial cells. According to the results of this study, the authors suggest the use of low-intensity electric current, glycerol and Tween 80 as cost-effective treatments or additives to increase the production of commercially neutral protease enzymes.

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