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Experimental Investigations on Behaviour of Rhamnolipid Biosurfactant as a Green Stabilizer for the Biological Synthesis of Gold Nanoparticles

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ABSTRACT

Use of biosurfactant as a green stabilizer for the biological synthesis of gold nanoparticles (AuNPs) is now emerging as a nontoxic and environmentally acceptable "green chemistry" procedure. Stability of AuNPs at different pHs is very important because our body has different pHs. This paper addresses this issue. In this work, first *P. aeruginosa* PTCC 13401 was used to produce rhamnolipid biosurfactant. The highest rhamnolipid production occurred at 120 h, achieving a value of 3.1 g/L. The thin layer chromatography (TLC) indicated that the crude product is a mixture of mono-rhamnolipid and di-rhamnolipid with retardation factor (Rf) value of about 0.35 and 0.78, respectively. Moreover, rhamnolipid solutions with different pHs were added to HAuCl4 solution and incubated for 24 h at 37 ^{o}C and 150 rpm. The formation of spherical AuNPs was monitored using a UV–vis spectrophotometer and verified by TEM. Our results showed that the formation of AuNPs occurred just for pH values between 7.0 -8.0. Measurement of the surface tension of the solution at different pH values was performed to find out the reason for this observation. Our results showed that the surface tension was also stable only between pH 7.0-8.0. This was inferred from precipitation of rhamnolipid at higher and lower pH values. The results of this work may help pharmacists to have a good prediction of behavior of rhamnolipid biosurfactants as a green stabilizer for biomedical applications including tissue engineering and drug delivery.

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1. INTRODUCTION

Recently, a range of nanoparticles have been extensively used for biomedical applications including tissue engineering, drug delivery, and biosensor. They have attracted interest because of their unique optical, thermal, electrical, chemical, and physical properties that are due to the large proportion of high-energy surface atoms compared to the bulk solid. They are widely applied in products that directly come in contact with the human body, such as shampoo, soap, detergent, and toothpaste, besides medical and pharmaceutical applications. Among the various nanomaterials, especially AuNPs have found use in diagnostic and drug delivery applications. AuNPs also have been reported for their antibacterial, anti HIV and anti tumor properties [1–5]. However, the use of chemicals in the synthesis of metal nanoparticles results in the release of toxic by-products which are hazardous to environment and humans. Therefore, there is a growing need to develop environmentally friendly processes for nanoparticle synthesis without use of toxic chemicals. The synthesis of metal nanoparticles using biosurfactants serves as a simple and eco-friendly alternative to chemical methods [5–8]. Biosurfactants are used in several industries including agrochemicals, fertilizers, foods, cosmetics, pharmaceuticals and many others due to their unique functional properties such as low toxicity. They can be used as emulsifiers, wetting agents, spreading agents, functional food ingredients and detergents [8–14]. The surface tension reducing ability of biosurfactants made them to play an important role in these industries. Literature survey on biosynthesis of

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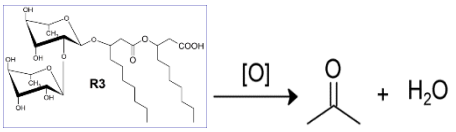
metal nanoparticles revealed that the produced biosurfactants by *Pseudomonas aeruginosa* (rhamnolipid) and *Bacillus subtilis* (surfactin) have been used extensively in this field. Husseiny et al. [15] and Rane et al. [16] reported that AuNPs were formed through reduction of gold ion by bacterial cell supernatant of *P. aeruginosa* and *Bacillus subtilis*. In another case, green production of AuNPs using a biosurfactant extracted from corn was investigated by Gómez-Graña et al. [17]. Also, an environmentally friendly method using a cell-free extract of *Rhodopseudomonas capsulata* was proposed for the synthesis of gold nanowires with a network structure by He et al. [18]. They controlled the shapes of gold nanoparticles with the change of $HAuCl_4$ concentration. Sriram et al. [19] described the methods for the intracellular biosynthesis of AuNPs using *Bacillus licheniformis*. Other authors such as Reddy et al. [20, 21] have also used surfactin as a stabilizing agent in the synthesis of AuNPs. According to previous research studies, stable AuNPs were formed by treating an aqueous $HAuCl_4$ solution using the biosurfactants as reducing agents (reduction of Au^{3+} ions). For example, it could be concluded that secondary alcohols in rhamnolipid molecules are converted to a ketone and oxidation happens through chemical reactions. This mechanism can be described by the following equation.



(1)

In other words, culture supernatant of *Pseudomonas aeruginosa* contains reductases, produced and secreted by the microorganism which is responsible for production of nanoparticles [22].

According to Rehman et al. [23] rhamnolipids serve as both reducing and stabilizing agents to produce gold nanoparticle. Figure 1 shows a picture of mechanisms for the biosynthesis of AuNPs. Despite the many efforts that have been made, it seems most of the reports about biological synthesis of gold nanoparticles from *P. aeruginosa* have just focused on operation conditions such as temperature, agitation speed and concentration of HAuCl4, and little work has been done on the behavior of produced AuNPs at various pHs. This issue for biomedical applications including tissue engineering and drug delivery is very important because our body has different pHs. The pH in our body may differ from one area to another with the highest acidity in the stomach (1.35 to 3.50). pH of blood ranges from 7.35-7.45. The skin is quite acidic (pH 4.0–6.5) to provide an acid mantle as a protective barrier to the environment against microbial overgrowth [24]. So the lack of detailed reports prompted us to investigate this gap.

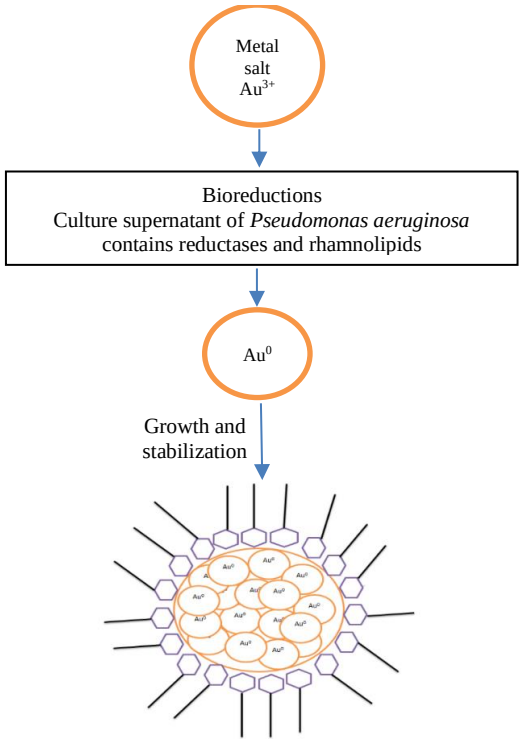


Figure 1. Mechanisms of biosynthesis of gold nanoparticles

2. MATERIALS AND METHODS

2. 1. Microorganism *P. aeruginosa* PTCC 13401 was purchased from the Persian Type Culture Collection, Tehran, Iran.

2. 2. Rhamnolipid Production In this study, Lactose broth (LB) solution was autoclaved at 121 °C for 20 min and then a loop of bacterium was added to 100 mL LB medium (pre-culture). The flask was then incubated at 37 °C in a shaker with an agitation speed of 150 rpm and (Mehr Tajhiz, Iran) and the bacterial growth was monitored over time until the culture reached OD_{600} 1.0. A production medium containing 100 g/L sunflower oil and a salt solution with 0.05 g/L $MgSO_4.7\ H_2O$, 1.5 g/L $NaNO_3$, and 0.1 g/L KCl; 0.1M sodium phosphate buffer at pH 6.5 was used throughout the study for the culture. A trace element solution (1 mL) consisted of the following composition: 2.0 g/L sodium citrate.$2H_2O$, 1.2 g/L $CuSO_4.5H_2O$, 1.4 g/L $ZnSO_4.7\ H_2O$, 1.2 g/L $CoCl_2.6\ H_2O$, 0.28 g/L $FeCl_3.6H_2O$ and 0.8 g/L $MnSO_4.H_2O$ was sterilized by filtration (0.22-µm) and added to the medium. The initial pH of the medium was adjusted to 6.5. A total of 5 mL from the pre-culture was incubated in the flask. Rhamnolipid production was carried out in a 1000 mL Erlenmeyer flask containing 100 mL of the above mentioned production medium at 150 rpm and 37 °C. For extraction of rhamnolipid, samples of the medium were taken for analysis at irregular time

intervals. Hexane was then added to each sample 1:1(v/v), and the samples were centrifuged at 4600 g for 20 min. After evaporation of n-hexane, sunflower oil concentrations were measured. For rhamnolipid measurement, an aliquot of the aqueous phase was acidified with 85% phosphoric acid 1:100 (v/v) to adjust the pH around 2–3, leading to precipitation of the rhamnolipids. Rhamnolipids were extracted twice with ethyl acetate 1:1.25 (v/v) [25].

2. 3. Identification of the Produced Biosurfactant by TLC and FTIR The type of the purified biosurfactant was identified by thin layer chromatography (TLC). According to Syldatk et al. [26], TLC was used to confirm the structure of the biosurfactant obtained from *P. aeruginosa* PTCC 13401. In this test, the samples at day 5 of cultivation were first extracted with hexane to remove the residual plant oil. Hexane was added 1:1 (v:v). After mixing at 4700 rpm and 4 °C for 10 min, the aqueous phase, the hydrophobic phase and the biomass were separated. Aqueous phase (lower phase) was subjected to further rhamnolipid analysis. TLC analysis was done on silica gel (60 F254, 0.25 mm, Merck) using chloroform-methanol-water 65:25:4 (v/v/v) as the solvent system. Spots were appeared by heating at 110 ºC for five minutes. FTIR spectroscopy was used, in ATR (Attenuated total reflectance) mode to identify the functional groups of the produced biosurfactant.

2. 4. Synthesis and Characterization of AuNPs Well grown (24 h) bacterial culture of *P. aeruginosa* PTCC 13401 was taken in a polypropylene tube and the bacterial cell pellets were collected by centrifugation at 5000 rpm at 25 ºC for 10 min. A 50 mL of supernatant with different pHs was added to 50 ml of 1mM $HAuCl_4$ solution and incubated for 24 h at 37 ºC and 150 rpm (reaction mixture was incubated until the colour changed). The pH of the supernatant was adjusted using 1 M HCl and 1 M NaOH solutions. The colour change for the $HAuCl_4$ solution from yellow to red was a visual confirmation of the reduction of Au^{3+}. Also the bioreduction of Au^{3+} and subsequent formation of AuNPs was characterized by UV-vis spectroscopy (Photonix Ar 2015). The UV-vis spectrum was recorded between 350-700 nm. Finally, the size and morphology of the biosynthesized AuNPs were visualized by a high resolution transmission electron microscope (TEM ZISS-EM900).

3. RESULTS AND DISCUSSION

3. 1. Time-course Profile of Batch Rhamnolipid Fermentation A typical time-course profile for batch rhamnolipid fermentation is shown in Figure 2. With an initial sunflower oil concentration of 120 g/L at 37 °C and an agitation rate of 150 rpm, the rhamnolipid concentration increased along with the cell growth, indicating that rhamnolipid was essentially a growth associated product. The highest biomass and rhamnolipid production occurred at 120 h, achieving a value of 7.7 g/L and 3.1 g/L, respectively. Moreover, sunflower oil concentration reduced from 120 to 2.1 g/L at the end of fermentation. Based on our research, *P. aeruginosa* PTCC 13401 displayed low productivity (3.1 g/L). Low productivity is still the major obstacle in the production of rhamnolipids [27]. Although the production of rhamnolipids is low, the benefits of using them are numerous and do not pose health risk. Therefore use of biosurfactants is absolutely necessary in medical applications.

3. 2. Characterization of Produced Biosurfactant As previously mentioned, production of rhamnolipid could be confirmed by TLC and FTIR analyses. Samples from the cell culture were taken at day 5 of cultivation. In this work, the two yellow spots observed on the TLC plate (Figure 3) were mono-rhamnolipid and di-rhamnolipid with retardation factor (Rf) value of about 0.35 and 0.78, respectively, and standard rhamnolipid gave the similar Rf values [27]. Therefore, it is quite reasonable to assume that the produced biosurfactant was rhamnolipid. In this research work, to determine the accuracy and repeatability, we made four spots in the TLC test. Also for further identification of the produced biosurfactant, FTIR analysis was carried out in the 4000-400 cm^{-1} spectral region. Based on Figure 4, the presence of rhamnose and long chain hydrocarbon was also confirmed by FTIR analysis. Figure 4 shows absorbance bands formed at 2926, 2857, 722 cm^{-1} and 840 cm^{-1} due to the C-H stretching of $-CH_2$ and $-CH_3$ groups and C-O stretching bands rising from ester and carboxylic groups were found at 1172 cm^{-1} and 1052 cm^{-1}. Similar results were also reported by Lan et al. [14] and Rikalovic et al. [28].

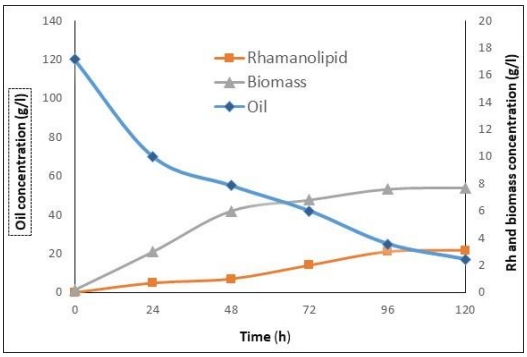


Figure 2. Profiles of cellular growth, rhamnolipid production and substrate consumption by *P. aeruginosa* PTCC 13401 (rpm = 150, temperature=37 °C and pH= 6.5)

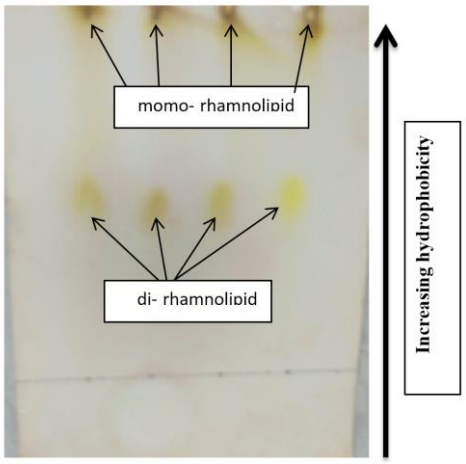


Figure 3. Results of TLC of produced rhamnolipid in this research. Specific spots were detected on TLC. Experiments were repeated 4 times.

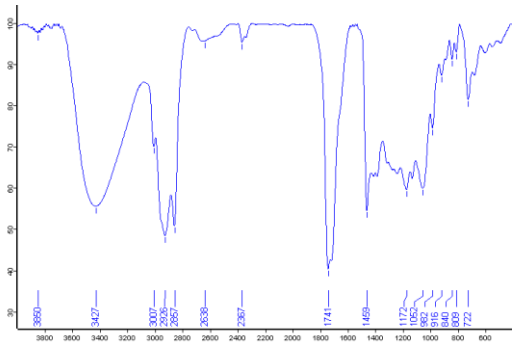


Figure 4. FTIR spectrum of rhamnolipid extracted from *P. aeruginosa* PTCC 13401

3. 3. Synthesis and Characterization of AuNPs

Colour photograph and UV-vis spectrum of AuNPs synthesized by cell-free supernatant of *P. aeruginosa* PTCC 13401 at pH=8.0 are depicted in Figure 5. As seen in Figure 5(a-i), the colour change for the $HAuCl_4$ solution from yellow to red within 24 h of incubation at 37 °C and 150 rpm provided an initial visual confirmation of the reduction of Au^{3+}. As shown in Figure 5(a-ii), the control experiment without rhamnolipid remained pale yellow, indicating the absence of AuNPs. The biosynthesis of AuNPs was further confirmed using UV-visible spectroscopy. Figure 5b shows the characteristic absorption spectrum related to the surface plasmon resonance (SPR) of AuNPs synthesized by cell-free supernatant of *P. aeruginosa* PTCC 13401. According to Figure 5b, AuNPs synthesized by this method showed a uniform and sharp peak at 540 nm which is characteristic surface plasmon of gold. Similar results were also reported by Rane et al. [16] and Das et al. [29]. Moreover, the biosynthesized AuNPs was characterized using TEM. From TEM image (Figure 6), it could be obviously found that the morphology of the gold nanoparticles is spherical and they are fairly uniform with an average size of ca. 53 nm. Based on the results of this study, it seems rhamnolipid biosurfactant could be used successfully for biosynthesis of AuNPs.

3. 4. Investigation of the Stability of AuNPs at Different pH Values

Stability of AuNPs at different pHs is very important in drug delivery and

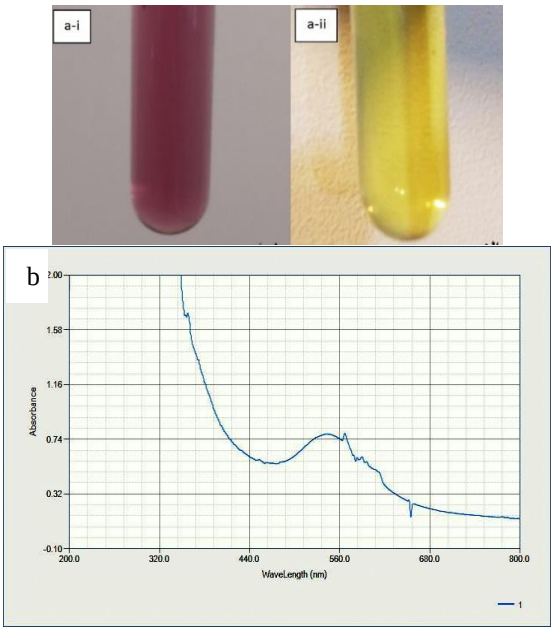


Figure 5. (a) Synthesis of gold nanoparticles using cell-free supernatant sample of *P. aeruginosa* PTCC 13401 at pH=8.0, where Figure 5(a-i) is the $AuHCl_4$ solution after 24 h incubation and Figure 5(a-ii) is the pure $AuHCl_4$ solution (control) and (b) surface plasmon resonance of AuNPs synthesized by cell-free supernatant from *P. aeruginosa* PTCC 13401.

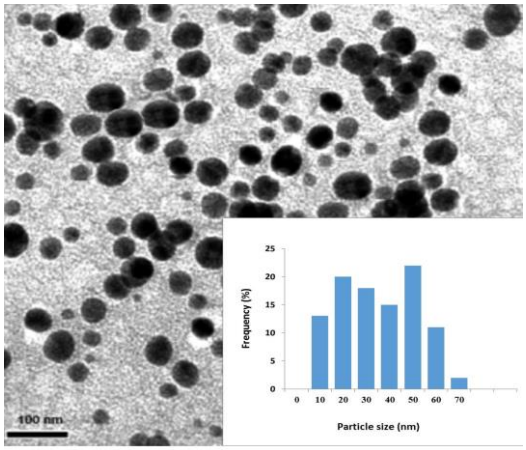
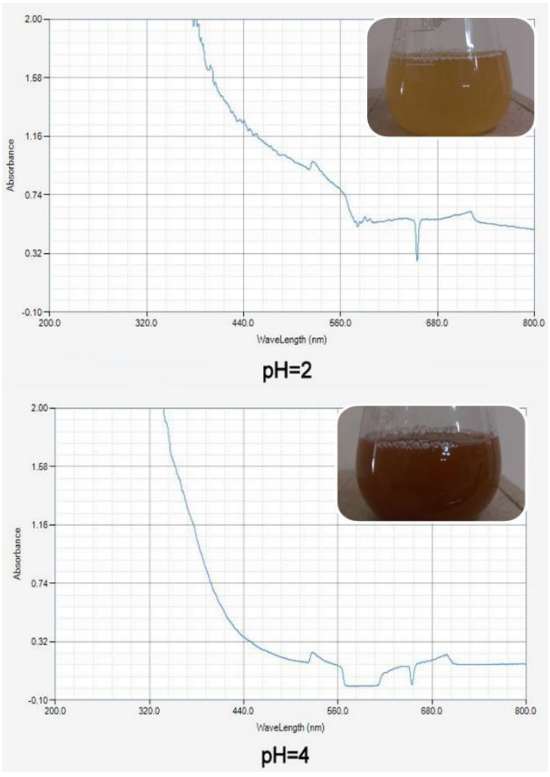


Figure 6. A typical TEM image of the AuNPs with size distribution histogram

biomedical applications because our body has different pHs. In this section, the effect of pH on green synthesis of AuNPs was investigated. Green synthesis of AuNPs was monitored by measuring the absorbance as shown in Figure 7. Through the graph, it was found that the maximum absorption peak of UV-vis spectrum was around 540 nm just for pH 8.0. To understand this, another test was carried out to find out the reason for this observation. For this, the surface tension of the produced biosurfactant (3.1 g/L) was measured at different pH values ranging from 2.0 to 10.0. In this part the impact of pH on the precipitation of rhamnolipids is invesigated by monitoring the surface tension as a result of changing the pH of solution. The results are shown in Figure 8. Based on this figure, the minimum amount of surface tension was about 28.0 mN/m only between pHs 7.0-8.0. Any solution with a pH lower than 7.0 and higher than 8.0 had a negative effect on surface tension. Similar findings have been reported by other researchers that the biosurfactant is precipitated by adjusting pH of the broth cell-free culture to 2.0 and 11.0 [30]. However, it seems that the failure of the biosynthesis of gold nanoparticles can be due to performance of rhamnolipids at high and low pH levels. Moreover, our stability studies showed that the produced green AuNPs were stable after 1 month at 37 °C and pHs 7.0-8.0. We used this temperature because the average normal body temperature is generally 37 °C. However, according to our results the



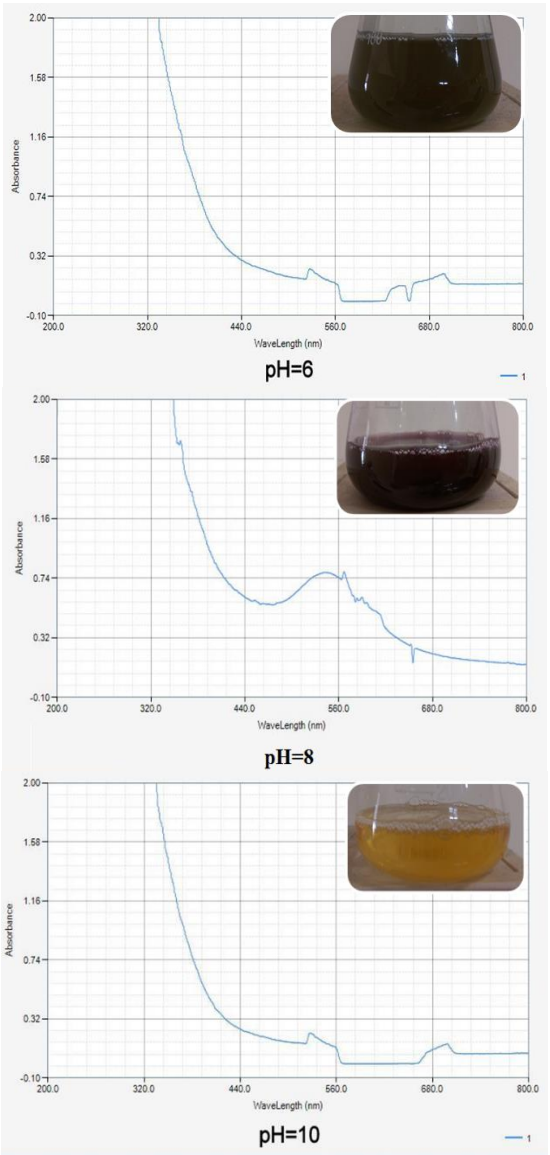


Figure 7. Effect of pH on the green synthesis of gold nanoparticles

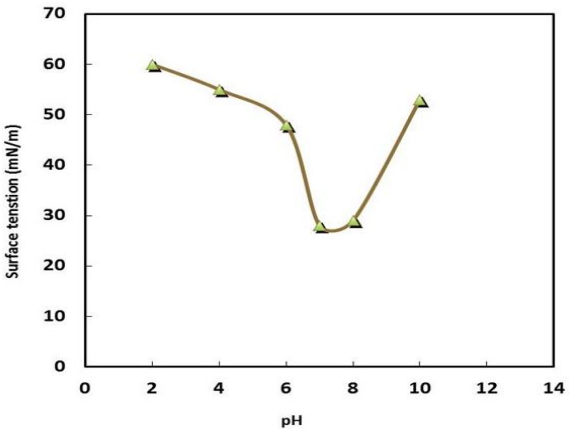


Figure 8. Effect of pH on rhamnolipid activity

produced green AuNPs can be used in pharmaceutical industry that use nanoparticles for the targeted delivery and controlled release of therapeutic agents (pH 7.0-8.0). The results of this work may help the pharmacists to have a good prediction of performance of the produced green AuNPs in medical applications.

4. CONCLUSIONS

Gold nanoparticles were successfully biosynthesized using rhamnolipids. The succesful synthesis of spherical gold nanoparticles was confirmed by UV and TEM analyses. Also the relationship between the biosynthesized of gold nanoparticles and the pH values of the solution was investigated. Our results showed that the gold nanoparticles were only biosynthesized at pHs between 7-8. A possible reason for this result was the precipitation of rhamnolipid at higher and lower pH values. These findings are confirmed by measuring the surface tension of produced rhamnolpid at different pH values. The results of this work may help the pharmacists to have a good prediction of biosynthesis of gold nanoparticles using rhamnolipids as one of the most widely used NPs.

5. ACKNOWLEDGEMENTS

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Persian Abstract

چکیده

امروزه استفاده از بیوسورفکتانت‌ها به عنوان یک تثبیت‌کننده سبز و غیرسمی برای ساخت بیولوژیکی نانوذرات طلا (AuNPs) در حال افزایش است. پایداری AuNPs در pHهای مختلف بسیار مهم است زیرا بدن ما pHهای مختلفی دارد. این مقاله به این مسئله می‌پردازد. در این کار تحقیقی، ابتدا از *P. aeruginosa* PTCC 13401 برای تولید بیوسورفکتانت رامنولیپید استفاده شد. بیشترین میزان تولید رامنولیپید پس از ۱۲۰ ساعت به مقدار ۳/۱ گرم در لیتر رسید. همچنین کروماتوگرافی لایه نازک (TLC) نشان داد که محصول ترکیبی از مونو رامنولیپید و دی رامنولیپید با Rfهای به ترتیب در حدود ۰/۳۵ و ۰/۷۸ است. علاوه بر این، محلول های رامنولیپید با pHهای مختلف به محلول $HAuCl_4$ اضافه شده و به مدت ۲٤ ساعت در دمای ۳۷ درجه سانتی‌گراد و ۱۵۰ دور در دقیقه انکوبه شدند. پس از این مدت، تشکیل نانوذرات طلای کروی با استفاده از طیف سنج UV-vis مورد بررسی قرار گرفت و توسط TEM تأیید نهایی شدند. نتایج این مقاله نشان داد که تشکیل نانوذرات طلا فقط برای مقادیر pH بین رخ داده است. برای یافتن دلیل این موضوع، اندازه‌گیری کشش سطح محلول‌ها در pHهای مختلف انجام شد. نتایج نشان داد که کشش سطحی نیز فقط بین pHهای ۷/۰–۸/۰ پایدار است که این می‌تواند به دلیل رسوب کردن رامنولیپید در مقادیر pH بالاتر و پایین باشد. نتایج این مقاله ممکن است به داروسازان کمک کند تا پیش‌بینی خوبی از رفتار رامنولیپید به عنوان یک تثبیت‌کننده سبز برای کاربردهای پزشکی مانند مهندسی بافت و رهایش دارو داشته باشند.