Enhanced oxygen transfer rate and bioprocess yield by using magnetite nanoparticles in fermentation media of erythromycin

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Abstract

Background: Magnetite nanoparticles have widespread biomedical applications. In the aerobic bioprocesses, oxygen is a limiting factor for the microbial metabolic rate; hence a high availability of oxygen in the medium is crucial for high fermentation productivity. This study aimed to examine the effect of using magnetite nanoparticles on oxygen transfer rate in erythromycin fermentation culture.

Methods: Magnetite nanoparticles were synthesized through co-precipitation method. After observing the enhanced oxygen transfer rate in deionized water enriched with magnetite nanoparticles, these nanoparticles were used in the media of by Saccharopolyspora erythraea growth to explore their impact on erythromycin fermentation titer. Treatments comprised different concentrations of magnetite nanoparticles, (0, 0.005, 0.02 v/v).

Results: In the medium containing 0.02 v/v magnetite nanoparticles, KLa was determined to be 1.89 time higher than that in magnetite nanoparticle-free broth. An improved 2.25 time higher erythromycin titer was obtained in presence of 0.02 v/v nanoparticles.

Conclusions: Our results, demonstrate the potential of magnetite nanoparticles for enhancing the productivity of aerobic pharmaceutical bioprocesses.

Keywords: Bioprocess, Oxygen transfer rate, Mass transfer coefficient (KLa), Magnetite nanoparticles, Pharmaceutical biotechnology, Fermentation, Erythromycin, Saccharopolyspora erythraea

Background

Oxygen is one of the most important substrate influencing productivity of aerobic bioprocesses [1]. While oxygen has a low solubility in most fermentation media, the uptake of the major amount of oxygen by microorganisms during the fermentation decreases the dissolved oxygen level in liquid to less than the critical concentration, rendering oxygen the limiting factor for productivity. A significantly low rate of oxygen transfer from gas into liquid, will lead to a decreased microbial metabolic rate, thereby low fermentation performance. Therefore an adequate supply of O₂ is required for achieving a high fermentation productivity, particularly in high-cell density bioprocesses [2] such as erythromycin fermentation by Saccharopolyspora erythraea.

The Oxygen Transfer Rate (OTR) is usually determined by volumetric mass transfer coefficient (KLa). This variable is affected by several factors, including composition of medium, and geometrical and operating characteristic of the bioreactor [3]. Several methods have been proposed for improving KLa, including use of more effective agitation and aeration systems, enriching air with pure oxygen, reducing gas bubbles’ size and enhancing gas hold up [2], and modifying physical properties of the medium by adding dispersed phases containing particles in size of μm [4] capable of solubilizing O₂ more than water.

Recent studies have shown that nanomaterials have potential to positively influence the variables affecting biochemical processes. For instance, Olle et al. [4] showed that O₂ mass transfer improves in the presence of colloidal nanoparticle dispersion. In addition, Nagi et al. [5]...
reported an enhanced oxygen mass transfer rate in the presence of nano-size particles.

Following this growing line of research, in the present study, first aqueous solution of magnetite nanoparticles (MNPs) was prepared. The solution was then added to the fermentation media of \textit{S. erythraea} to examine the possible effects of magnetite nanoparticles on \textit{O}_2 transfer rate and final titer of fermentation product.

\textbf{Methods}

\textbf{Materials}

The erythromycin-producing strain \textit{Saccharopolyspora erythraea} PTCC 1685 was obtained from Persian Type Culture Collection I-124, Iran. Soybean flour was supplied from Maxsoy Co., Iran. Chemical reagents and media were purchased from Merck or Sigma.

\textbf{Synthesis of nanoparticles}

Several procedures have been developed for synthesis of iron oxide nanoparticles [6,7]. In this study, co-precipitation technique was used for synthesis of magnetite nanofluid, which is based on the simultaneous precipitation of \textit{Fe}^{3+}, \textit{Fe}^{2+} ions in basic aqueous media [6]. Some advantages of this method include being straightforward, cheap, and environment-friendly, and producing a uniform size distribution of nanoparticles.

To prepare solution of MNPs, 23.5 g \textit{FeCl}_3·6\textit{H}_2\text{O} and 8.6 g \textit{FeCl}_2·4\textit{H}_2\text{O} were dissolved in 25 ml deionized water in an Erlenmeyer flask under Nitrogen sparging, 80°C and vigorous mechanical stirring. After 30 min, 45 ml of an aqueous solution of \textit{NH}_4\text{OH} was added to the mixture dropwise, after which the color of solution changes from light-brown to black, an indication of MNPs formation. The reaction was continued at 80°C under stirring and Nitrogen sparging conditions for 30 min to allow the substance completely crystallize. Afterwards the solution was cooled at room temperature [4,8-10]. The process reaction follows the following formula:

\[ \text{FeCl}_3 + 2\text{FeCl}_3 + 8\text{NH}_3 + 4\text{H}_2\text{O} \xrightarrow{1200 \text{ rpm}} \text{Fe}_3\text{O}_4 + 8\text{NH}_4\text{Cl} \]

Due to their strong magnetic properties, the synthetic MNPs were aggregated near the magnet. MNPs were then washed with deionized water three time sat the end of process. Next, NPs were dried in oven at 80°C overnight to be characterized by XRD and TEM analysis [4].

\textbf{Experimental determination of the volumetric mass transfer coefficient (KLa) by dynamic method}

According to the Dynamic method [11] for determining mass transfer coefficient, first the concentration of the dissolved oxygen in the liquid phase in reduced by means of Nitrogen bubbling until the oxygen concentration falls to zero. Afterwards, by bubbling the air into the reaction container the concentration of dissolved oxygen is increased [11-13]. The KLa can then be calculated using the two-film theory. According to this theory, the rate of oxygen transfer from gas phase into liquid phase (at cell-free systems) is represented by:

\[
\frac{dc}{dt} = K_{La}( C^* - C_L)
\]

where \(dc/\text{dt}\) is the accumulation rate of the oxygen in the liquid phase, \(K_{La}\) represents the lumped volumetric mass transfer coefficient, \(C^*\) denotes the saturate concentration of the dissolved oxygen in the broth, and \(C_L\) represents the dissolved oxygen concentration in the aqueous phase [14]. Integrating Equation 1 will result in Equation 2:

\[
\int_{C_{L1}}^{C_{L2}} \frac{dC_L}{C^* - C_L} = \int_{t1}^{t2} K_{La} \cdot dt
\]

With \(CL1 = 0\) at \(t1 = 0\), the integrated form of Equation (2) can be represented as the Equation 3:

\[
\ln \frac{C^* - C_{L2}}{C^* - C_{L1}} = -K_{La} \cdot t_2
\]

A plot of \(\ln \frac{C^* - C_{L1}}{C^* - C_{L2}}\) vs. \(t\) will result in a straight line with slope of -KLa [12,14].

\textbf{Media and cultural method}

Spores of \textit{S. erythraea} were produced on slants of CSL agar medium after 14 days in incubator at 30°C [15]. Ingredients of the sporulation medium used in this study was (per liter): 10 g CSL, 10 g starch, 2.5 g \textit{CaCO}_3, 3 g (\textit{NH}_4)_2\text{SO}_4, 3 g \text{NaCl}, 2 ml trace element (\textit{MgSO}_4·7\textit{H}_2\text{O}, \textit{FeSO}_4·7\textit{H}_2\text{O}, \textit{ZnSO}_4·7\text{H}_2\text{O}, \textit{CuSO}_4·5\text{H}_2\text{O}, \textit{CoCl}_2·6\text{H}_2\text{O}, \text{HCl 37%}), pH 7 ± 0.1 [15]. A volume of 1 ml of spore suspension of strain was inoculated in a 1000 ml Erlenmeyer flask containing 100 ml of seeding medium and incubated at 30°C and 220 rpm for 48 hours on a shaker-incubator. The composition of seeding medium used in this study was (per liter): 30 g soybean meal, 10 g glucose, 10 g glycerol, 3.5 g (\textit{NH}_4)_2\text{SO}_4, 1 g (\textit{NH}_4)_2\text{HPO}_4, 5 g \text{CaCO}_3, pH 7 ± 0.1 [15]. Based on strain’s morphology (Figure 1), culture’s pH and biomass, the best inoculum was selected to be inoculated (5% v/v) into fermentation flasks. Fermentations were carried out in 9 Erlenmeyer flasks of 1000 ml, containing 150 ml of fermentation media. The composition of fermentation media was (per liter): 30 g soybean meal, 40 g dextrin, 30 g starch, 2 g(\textit{NH}_4)_2\text{SO}_4, 0.15 g (\textit{NH}_4)_2\text{HPO}_4, 10 g \text{CaCO}_3, 50 g rapeseed oil, pH 6.8. Two concentrations of MNPs (0.005, 0.02 v/v) were added to each fermentation flask. Flasks were then put in shaker-incubator at 33°C.
and 220 rpm for 11 days [15-17]. All experiments were performed in triplicate in six batches. Samples of 7 ml were taken on days 6, 8, and 10 for further analysis. Investigation of *S. erythraea* morphology indicated that the hyphae of the strain were lysed on day 12, hence, no further erythromycin production was occurred.

**Erythromycin assay**

Purification of erythromycin was carried out by centrifugation of the samples (4000 rpm, 20 min) followed by using magnet to separate the possibly remaining magnetite nanoparticles from the supernatant. The supernatant was then diluted with 0.2 M carbonate-bicarbonate buffer of pH 9.6 and the total erythromycin was extracted with chloroform. The extracted erythromycin was then mixed with the bromophenol blue reagent. The absorbance of organic phase was measured at 415 nm by spectrophotometry [15-17].

**Statistical analysis**

T-test was used to examine the significance of the mean differences. KLa was calculated from regression analysis. P < 0.05 was considered as the statistical significance.

**Results and discussion**

**Nanoparticles characterization**

**NPs’ crystal structure**

The crystal structure of the synthetized MNPs is characterized by X-ray powder diffraction (XRD) [9]. Figure 2 displays the structural properties of the MNPs as determined by XRD (Bruker axs D4, cu, step size 0.02). The XRD pattern shows the diffraction peaks at $2\theta = 18.270^\circ$, $30.035^\circ$, $35.423^\circ$ (strongest line), $43.053^\circ$, $53.392^\circ$, $56.944^\circ$, $58.059^\circ$, $68.754^\circ$.
Figure 4 The size distribution of magnetite nanoparticles.

Mean=11.24
SD=3.5375
N=300

Figure 5 The dissolved oxygen at different concentrations of MNPs (each value in the figure is the mean of corresponding values from 3 repeated experiments).
62.516°, which correspond to the (111), (220), (311), (400), (422), (511), (440) reflections, respectively [9,18-20]. The XRD results show that the synthesized MNPs are well crystallized and the relative intensity of the diffraction peaks matches well with the reported XRD data for magnetite nanoparticles in the literature.

**NPs’ size**
Morphology and size of the prepared MNPs was determined by Transmission Electron Microscopy (TEM) (Figure 3) [10,21-23]. A small amount of dried MNPs were dissolved in ethanol. The solution was then sonicated for better dispersion. A drop of the solution was then placed on a 200 mesh Copper grid and dried in the air. The sample was analyzed by TEM at 100 KV.

Diameter of the MNPs was determined directly from the TEM image [24]. Figure 4 displays the histogram of NPs’ size distribution ($n = 300$). A mean (SD) size of particles is 11.24 (3.5) nm was calculated.

**Effect of nanoparticles on mass transfer coefficient**
The changes in concentration of dissolved oxygen in deionized water at different concentrations of MNPs were measured during the absorption process, based on Dynamic method [11,12], and compared with nanoparticle-free water, as the control. As shown in Figure 5, at all concentrations of MNPs, the amount of DO has increased and the time for achieving saturate concentration has decreased, when compared with the control. $K_La$ was determined using the linearized curve of DO vs. time (see Methods). As shown in Figure 6, MNPs positively

\[
\begin{align*}
&y = -0.053x + 0.4183 \\
&R^2 = 0.94 \\
&y = -0.0698x + 0.3261 \\
&R^2 = 0.96 \\
&y = -0.0977x + 0.6501 \\
&R^2 = 0.96
\end{align*}
\]

-9
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
0 1 0 0 2 0 3 0 4 0 5 0 6 0 7 0 8 0 9
Time (min)

Figure 6 The linearized curve of dissolved oxygen at different concentrations of MNPs (the slope of the curve represents $K_La$).

**Figure 7** Effect of different concentrations of nanoparticles on the production of erythromycin by *S. erythraea* (each value on the figure is the mean of corresponding values from 12 repeated experiments).
affects the mass transfer coefficient; KLa in water containing 0.02 v/v MNPs is 1.85 times higher than that in the control.

Effect of MNPs on erythromycin titer
Magnetite MNPs with initial concentrations of zero (control medium), 0.005, 0.02 v/v were used in fermentation media of S. erythraea and their effect on erythromycin titer was examined. As shown in Figure 7, while the maximum concentration of erythromycin is 18.1 mg/lit in the control medium, in the medium with 0.02 v/v MNPs, a maximum titer of 32.86 mg/lit has obtained which is 2.25 times higher. Erythromycin final titer (day 10) in both 0.005 and 0.02 (v/v) media shows significant difference as compared with the control (P < 0.05). Figure 6 shows that this higher titer is due to the more prolonged production of erythromycin in MNP-containing medium compared to the control medium. It could be argued that this higher titer is due to an extended viability of the microorganism as a result of higher OTR in the presence of MNPs.

The chief objective of the present study was to explore the impact of MNPs on OTR and thereby titer of bioprocess product. MNPs are strong oxygen absorbent due to their increased surface area at nano scale. These nanoparticles have proven useful when used as recyclable oxygen carriers in aerobic fermentation [25]. A number of mechanisms proposed for the positive impact of MNPs on OTR include enhancing oxygen solubility in the fermentation medium, inducing microconvection in the surrounding fluid by Brownian motion [4], and enlarging the gas-liquid interfacial surface through being adsorbed on the air bubbles, preventing them from coalescence [26].

Results obtained in this study, which are in close agreement with the findings from previous researches [4,25–27], add weight to the notion of MNPs being biocompatible, non-toxic and non-volatile nanoparticles, together with their positive impact on oxygen transfer rate, introduce them as promising agents for achieving an enhanced productivity of the industrial bioprocesses.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
HA and AH jointly conceived and designed the study and contributed to the revision of the manuscript. GL made the major contribution to performing the experiments, summarizing and interpretation of the results, and drafting the manuscript. AR was involved in statistical analyses and drafting the manuscript. SMRS coordinated the study procedure and contributed to revision of the manuscript. All authors read and approved the final manuscript.

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