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Full Length Research Paper

Purification of rhamnolipid using colloidal magnetic nanoparticles

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Phospholipid-coated colloidal magnetic nanoparticles with mean magnetite core size of 9 nm are shown to be effective ion exchange media for the recovery and purification of Rhaminolipid from culture mixtures. These particles have high adsorption capacity for purification (an order of magnitude larger than the best commercially available adsorbents) and exhibit none of the diffusion resistances offered by conventional porous ion exchange media. Furthermore, purification in biological processes using colloidal magnetic nanoparticles results in saving the cost and time. In this study, production of the Rhaminolipid by *Pseudomonas aeruginosa* in culture media (MSM) with two types of carbon sources was carried out. Then, purification analysis was done to two types of solutions: 1. culture media without the strain 2-culture media with the strain at the different conditions of pH and ionic strength. The results of this purification method were compared to the results obtained of TLC purification method. Finally, purification of the Rhaminolipid was determined over 90% by this method.

Key words: Nanoparticle, rhaminolipid, purification, ***Pseudomonas aeruginos***, separation.

INTRODUCTION

Adsorptive and chromatographic separation processes play a fundamental role in a wide range of biological, analytical, and environmental (Rousseau, 1987). The expansion of adsorptive techniques and methods for the separation and purification of bio-molecules, particularly, has been a basic enabling power in the evolution of the biotechnology industry over the past few decades by Stephanopoulos (1993). Yet, despite the remarkable progress that has been made in these ways, there is still considerable area for inventive methods for the separation of bio-molecules from the milieux in which they are produced, especially with the common interest in larger molecular weight. Bio-products such as nucleic-acids and viral vaccines.

Most adsorptive processes for purification, containing ion exchange, affinity, hydrophobic and reverse phase chromatography are accomplished in packed beds of particles typically having about tens of microns diameters (Janson and Ryden, 1989).

Packed beds are generally characterized by high-pressure drops, however, cannot control “dirty” feed streams including plenty of particulate materials such as cell debris or other colloidal contaminants that can result in column plugging by Scopes (1987). These difficulties can be overcome either by expanded bed systems, in which the particles are fluidized by the up flowing feed solution, or by floating the particles in stirred batch systems and recovering them from the feed stream by micro filtration or dial filtration once the particles have been loaded with the target species .

The filtration itself can be slow because of low membrane fluxes, and the presence of the particulate contaminants can lead to obstruction of the membranes, therefore reduce their efficacy and lifetimes. In most cases, adsorbent beads are produced with large internal porosities to ensure sufficient surface area for adsorption, and most of the time adsorption processes can be slowed by the diffusional restrictions within the particles, in particular for macrosolutes such as DNA and viruses. The capacity of the beads can also be greatly restricted when large solutes are targeted because the larger pore sizes needed to allow penetration of these solutes into the porous matrix necessarily lead to a significant decrease in the active surface area per unit volume of matrix. Besides, the short contact times in expanded bed

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systems mean that only the external particle surfaces are available for bio-products such as rhamnolipid capture, and hence these systems, too, have capacity restrictions.

It is clear that there are a lot of motivations to develop new adsorptive separation media to provide large interfacial areas, small diffusion resistances, and high selectivity and capacity for large bio-products, media that recovered and regenerated easily, even in the presence of colloidal contaminants.

We investigate here for a way to satisfy these criteria using specially prepared nanoparticles coated with self-assembled layer of surfactants to provide an efficacious environment for the recovery and/or separation of materials from dilute solutions. Such particles have high surface areas per unit volume and minimal transport resistances owing to the small diffusion distances between particles. Their surfaces can be improved to provide the necessary affinity for the desired solutes and to provide stabilization of the colloidal suspension.

In addition, such colloidally dispersed nanoparticle adsorbents are mobile and hence can accommodate large colloidal materials within their suspension without concerning about the plugging associated with more conventional packed bed technologies. Therefore, making them suitable for controlling dirty systems with suspended cells and cell debris in addition to other colloidal contaminants or impurities.

While such particles have been contacted with the feed solution, they must be captured and regenerated for subsequent reuse, and the solute must be recovered. This capture can be performed through ultra filtration, or the particles can be produced to agglomerate to form larger flocs that can be micro filtered or centrifuged. These approaches can be mistaken if there are other nano or microscopic materials present, such as particulates or cell debris, and it would be advantageous to use another way to gain the particles. The approach we have investigated in this work is to use magnetic fluid nanoparticles that can be recovered by using high-gradient magnetic separation technology (Kelland, 1998). These particles are single domain magnetic dipoles that, in the lack of an applied magnetic field, show no preferred directional ordering, since the magnetic dipole-dipole interactions are weak (<1 kT), and thermal forces dominate the spread of these particles. When an enough high magnetic field gradient is applied, the particles show a preferential ordering in the direction of this field, which also exerts a force on the particles, which can be approximately calculated by Rosensweig (1985).

$$F_m = \chi V_{core} \mu H \nabla H \quad (1)$$

Where χ and V_{core} are the magnetic susceptibility and volume, respectively, of the particle core, μ is the magnetic permeability of free space, and H is the magnetic field. It is this magnetic force that is exploited in the capture of the particles. Generally, even with fields on the order of 2 T, the gradients established across the flow channel are inadequate to keep the particles and hold them against the diffusive and convective drag forces within the column. In order to overcome this restriction, the channel is packed with a mesh of magnetizable steel wool that dehomogenizes the magnetic field by distorting the field lines near the wire surfaces, supplying a high magnetic field gradient density that can be exploited to trap the nanoparticles and hence to filter them from the feed solution.

Other explorer have explored the use of magnetic particles in separations (Hatch and Stelter, 2001; Khng et al., 1998; Obrien et al., 1996), but these beads have generally consisted of small magnetic particles embedded within a polymer matrix and have been all considerably larger (Khng et al.,1998; Obrien et al., 1996) (by 1 - 3 orders of magnitude) than those considered here and are therefore more restricted in the specific areas available for adsorption and in the access to the inner surfaces within the porous particles.

The purpose of this paper is to report on production of rhaminolipids by a ***Pseudomonas aeruginosa*** and the synthesis of magnetic nanoparticles of the type reported by Lifen Shen, Paul E. Laibinis, and T. Alan Hatton (Shen et al., 1999) and on their use in the adsorptive separation of model Rhamnolipid by ion exchange processes. The loaded particles are recovered by magnetic field in order of 2 T and regenerated by either pH swings or exposure to high ionic strengths.

This process bases on two phases, First step: production of rhamnolipid from pseudomonas bacteria. Second step: synthesis of magnetic nanoparticles and purification of rhamnolipid by synthesized nanoparticles (Rashedi et al., 2006; Rashedi et al., 2006).

Pseudomonas species is well known for its ability to produce rhamnolipid biosurfactants with potential surface active properties when grown on different carbon substrates (Patel and Desai, 1997; Mercade and Manresa, 1994) and therefore is a promising candidate for large scale production of biosurfactants. Four different rhamnolipid homologues, produced by ***P. aeruginosa***, have been identified and characterized (Itoh et al., 1971; Para et al., 1989) (Figure 1). The rhaminolipids consist of one or two L-rhamnose units and one or two units of ß-hydroxydecanoic acid. RL 1 and RL 3 are the principal rhaminolipids produced. RL 2 and RL 4 are biosynthesized only under certain culture conditions.

MATERIALS AND METHODS

Bacteria and culture conditions

P. aeruginosa that used as parent strain was kindly provided Persian Type Culture Collection (PTCC). The strain was confirmed by PTCC Identification report No. 1011 as ***P. aeruginosa***, therefore

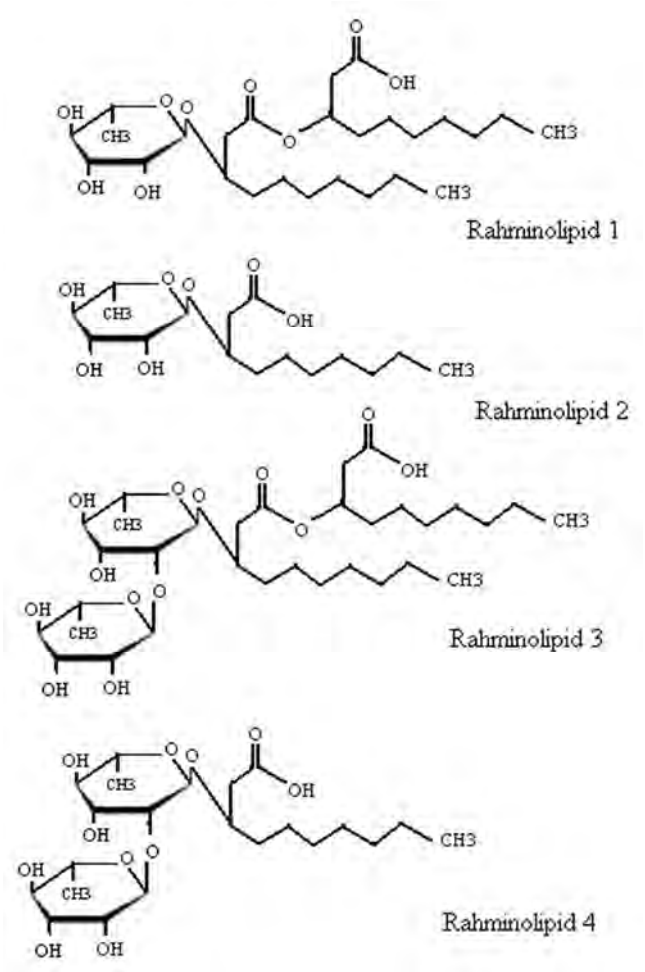

Figure 1. Structure of Rhamnolipid.

has been designated as ***P. aeruginosa*** MM1011 (Rashedi et al., 2006; Rashedi et al., 2006).

The strain was maintained on nutrient agar slants at 4°C and sub cultured every two weeks. Every 3 months a new frozen culture has been used to provide slant cultures. These frozen stocks were prepared by transferring a loop of stock culture to a 250 - ml Erlenmeyer flask containing 50 ml of mineral salts medium and 2% (v/v) corn oil as carbon source. After growing in shaker incubator at 30°C and 200 rpm for 3 days, 30 ml sterile glycerol was added and mixed thoroughly. Then, 2 ml of solution were dispensed into two sterile vials and stored at -70°C. Frozen cultures were recovered by thawing at 30°C to 25 ml of sterile mineral slats medium containing 25% (v/v) corn oil as carbon source and another one containing glucose as carbon source and incubated on a shaker incubator at 30°C for 3 days. ***P. aeruginosa*** MM1011 was grown in 500 ml erlenmeyer flasks containing 6% glucose as carbon source and 100 ml of the sterile mineral salts medium at 200 rpm and 30°C for 12 h. The mineral salts medium used throughout this study was made according to Lindhardt et al. (1989), contained (gL^{-1}): $NaNO_3$, 15; KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; yeast extract 0.5; and 5 ml trace elements solution containing (g L^{-1}): $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4.\ 5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17.

The trace element solution was filter-sterilized through a 0.22 µm filter (Millipore, type GS) and was added to the medium which had been autoclaved and then allowed to cool.

Production of bio surfactants

The parent strain of ***P. aeruginosa*** MM1011 was grown aerobically in 1 L Erlenmeyer flasks containing 200 ml of the mineral salts medium (described above) at 200 rpm and 37°C. Culture samples (3 ml) were drawn periodically for biomass and biosurfactant quantification under aseptic conditions. The total viable count were determined by the pour plate method,using plate count agar as the medium. In this process, the rhaminolipids produced by ***P. aeruginosa*** were used to prepare the sample mixture and study the effects of pH and ionic strength on purification of rhaminolipids by magnetic nanoparticls.

Synthesis of magnetic nanoparticles

Iron (II) chloride tetrahydrate (99%), iron (III) chloride hexahydrate (97%), ammonium hydroxide (28% NH_3 in water, double distilled), nonanoic acid (96%), decanoic acid (99%), acetone and methanol were purchased from Merk. DMPG (1,2-myristoyl-sn-glycero-3-phosphoglycerol, sodium salt) and DMPC (1,2-myristoyl-snglycero-3-phosphocholine) were purchased from Genzyme Pharmaceuticals. All chemical were used as received.

Preparation of magnetoliposomes

Fe_3O_4 nanoparticles coated with a decanoic acid surfactant bilayer were synthesized by chemical co precipitation from an aqueous solution of Fe (II) and Fe (III) salts in the presence of molecularly dispersed surfactant in a basic medium. The detailed synthetic procedures can be found elsewhere (Shen et al., 1999). To generate magnetite-phospholipids complexes, a mixture of surfactant-stabilized magnetic nanoparticles (2 mL of a 2.5 wt % solution) and phospholipids vesicles of DMPG and/or DMPC (10 mL of a 10 mM solution) was dialyzed in Visking Tubing (30/32) for 48 h against a buffer solution; this outside buffer was changed after 24 h. The phospholipids vesicles were prepared by sonication with a probe-type sonic disintegrator (Branson Sonifier 450) in 5 mM TES buffer until the solution was clear.

This procedure resulted in the removal of the outside, secondary surfactant layer as it desorbed and reported to the buffer solution on the other side of the dialysis membrane; this surfactant layer was replaced by a secondary layer of the phospholipids supplied by the phospholipids vesicles in suspension. The sizes of the particles were determined by electron microscopy (TEM) (model zeiss – CEM 902A operated at 220 kv).

Suspension stability

The stability of the nanoparticle suspensions against variations in ionic strength was determined by adding sodium chloride (NaCl) to 5 - ml aliquots of 0.07 wt% magneto liposome solution to make up 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.75 and 1 M NaCl concentrations in the medium. To test the pH stability, the pH of 5 ml aliquots of 0.07wt % magneto liposome solution was adjusted to be 2, 3, 4, 10, 11 and 12. Temperature stability was assessed by cooling 0.07 wt % magneto liposome solutions to 2°C and heating them to 80°C. In all cases, the suspension was deemed to be stable if no precipitation of the particles was observed over a 24 - h period under the test conditions.

Purification experiments

Various concentrations of rhaminolipid in two types of solutions were mixed with magnetoliposomes and incubated for 2 h at room

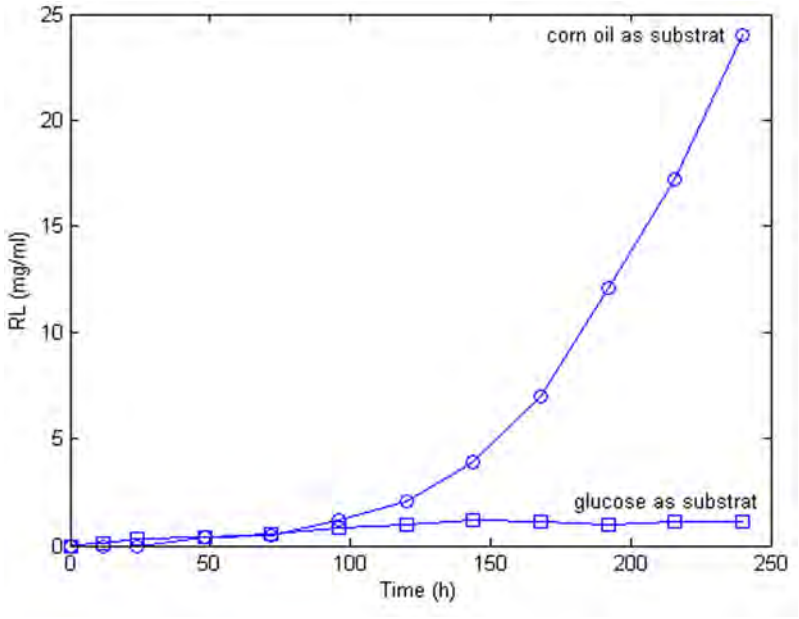

Figure 2. Time profiles of the production of the rhamnolipid (mg L^{-1}) by ***P. aeruginosa*** MM1011.

temperature to allow sufficient time for adsorption and stabilization of the solution. These two types mentioned above were:

i) The solution containing rhaminolipid, mineral salts medium (culture conditions of ***P. aeruginosa***) and Milli-Q water,
ii) The solution containing rhaminolipid, mineral salts medium (culture conditions of ***P. aeruginosa***), bacteria (***P***. ***aeruginosa***) and Milli - Q water.

A magnetic field (2 T) was used to separate the phases and the supernatant of the solution was withdrawn for analysis.

The experiment was done in various rhaminolipid concentrations (0.25 to 7.0 mm) at different pH values (pH 2 to 7), different ionic strengths (determined by adding sodium chloride (NaCl) to solution to make up 0.0, 0.01, 0.5, 0.1 and 0.15 Mm NaCl concentrations in the medium). The percentage of rhaminolipid purification was determined by purify the concentration in supernatant after impressing magnetic field and comparing it with the results were obtained by TLC purification method.

RESULTS AND DISCUSSION

Rhaminolipid production

The result of growing process of ***P. aeruginosa*** and production of rhaminolipid in mineral salt medium containing corn oil and glucose as a substrate is shown in Figure 2 and Tables 1a and 1b. In medium containing corn oil, growth of the bacteria will be reached. The stationary state after 3 days, and the number of active bacteria per 1 ml of culture medium left constant. However, the production of rhaminilipid was continued consistently, within using glucose as a substrate amount of rhaminolipid production is less and in comparison with corn oil. The process of growing of strain is more by this reason and the time to reach in a constant state of growing is longer and in the stationary state there is more strain in 1 ml of culture conditions. Corn oil and glucose as two carbon sources have been used to produce rhaminolipid.

Characterization of magnetoliposomes

The phospholipid-coated particles synthesized in this work possessed structural characteristics and properties similar to those of the surfactant-coated magnetite nanoparticles reported in previous communications (Shen et al., 1999; Shen et al., 2001).

We discuss here only the size information that is of direct relevance to adsorption processes, as this topic is the primary thrust of the current work. The interested reader is referred to our earlier papers (Shen et al., 1999; Shen et al., 2001) for a complete accounting of other properties of such magnetic fluids.

A TEM image of the magnetoliposomes is shown in Figure 3, where the particle core size is seen to be approximately 9 nm, with some poly dispersity that is characteristic of magnetite particles prepared by the precipitation method. The crystal structure evident in this figure confirms that the particles are single-domain crystals.

Characterization of magnetoliposomes

The phospholipid-coated particles synthesized in this work possessed structural characteristics and properties

Table 1a. The typical growth and bio-surfactant production of the parent strain ***P. aeruginosa*** MM1011 using corn oil as substrate.

Time (h)	TVC (cfu ml^{-1})	ST (mNm^{-1})	RL (mg ml^{-1})
0	1.15E+09	70.818	0
12	3.50E+09	65.12	0
24	1.10E+10	56.98	0
48	3.60E+10	45.584	0.43
72	4.60E+10	34.188	0.51
96	4.90E+10	28.49	1.21
120	4.70E+10	27.676	2.05
144	5.70E+10	27.676	3.92
168	5.35E+10	27.676	6.99
192	4.65E+10	27.676	12.1
216	5.15E+10	27.676	17.22
240	5.30E+10	27.676	24.03

TVC: Total Viable Count
ST: Surface Tension
RL: Rhaminolipid

Table 1b. The typical growth and bio-surfactant production of the parent strain P. aeruginosa MM1011 using glucose as substrate.

Time (h)	TVC (cfu ml^{-1})	ST (mNm^{-1})	RL (mg ml^{-1})
0	2.36E+09	71.63	0
12	1.76E+10	68.38	0.12
24	3.98E+10	61.83	0.32
48	5.60E+10	52.1	0.43
72	5.98E+10	39.07	0.53
96	5.20E+10	32.56	0.82
120	5.98E+10	30.93	0.99
144	7.90E+10	29.93	1.232
168	8.25E+10	29.3	1.132
192	7.90E+10	29.3	1.02
216	4.80E+10	27.68	1.16
240	5.00E+10	29.3	1.15

TVC: Total Viable Count
ST: Surface Tension
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similar to those of the surfactant-coated magnetite nano-particles reported in previous communications (Shen et al., 1999; Shen et al., 2001). We discuss here only the size information that is of direct relevance to adsorption processes, as this topic is the primary thrust of the current work. The interested reader is referred to our earlier papers (Shen et al., 1999; Shen et al., 2001) for a complete accounting of other properties of such magnetic fluids.

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The size and size distribution of the colloidal particles in the magnetic fluids were characterized by MALVERN Instruments Nano-Zs-zetasizer that have been shown in Table 2 and Figure 4 respectively. The average hydro-dynamic diameter of the particles is 35.6 nm.

Colloidal Stability of nanoparticle Suspensions

The nanoparticle suspensions were observed to be stable (that is, no observed precipitation after 24 h of exposure) at salt (NaCl) concentrations up to 0.5 M and

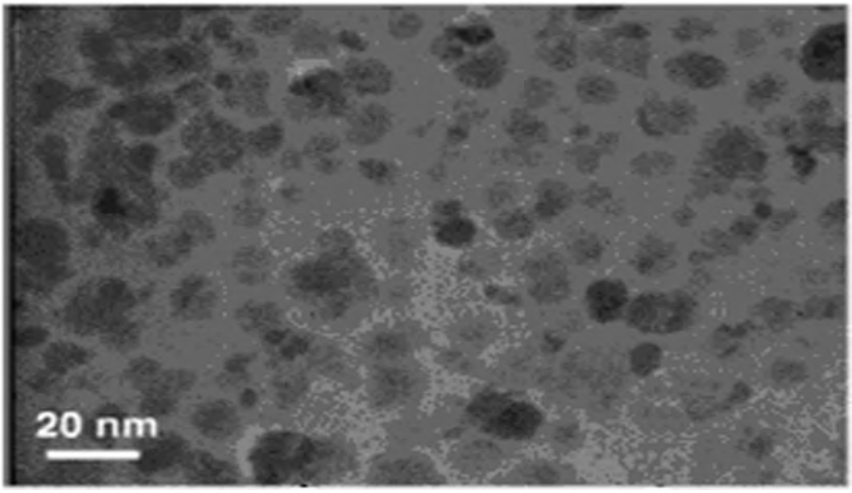

Figure 3. Transmission electron microscopy (TEM) images of Coated magnetic nanoparticles. The particles are polydisperse, contain single - domain magnetite crystals, and have an average Particle size of about 9 nm.

Table 2. Particle size distribution.

Peak	Diameter (nm)	% Intensity	Width (nm)
Peak 1	35.6	100.0	18.4
Peak 2	0.00	0.00	0.00
Peak 3	0.00	0.00	0.00

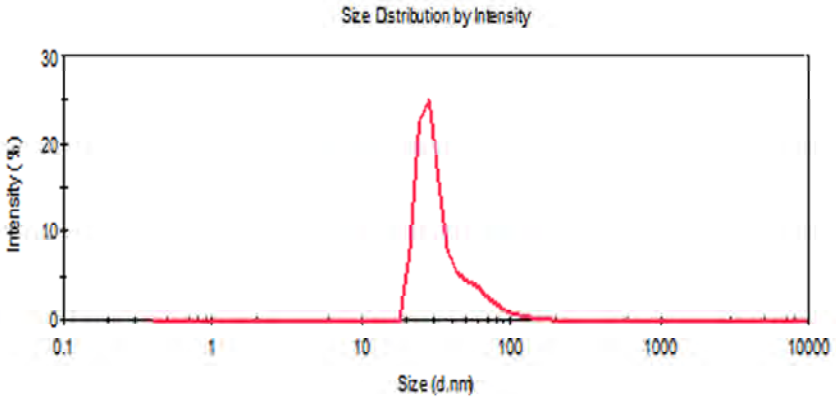

Figure 4. Size distribution of the colloidal particles in the magnetic fluids.

to a pH range of 3.5 - 12. No changes in suspension properties were noted at either 2 or 80°C. Stable suspensions of up to 15% wt /vol were readily prepared with no sign of the precipitation.

Ionic strength effects

The effect of ionic strength on the purification Rhamnolipid by DMPG coated nanoparticles at neutral pH is shown in Figures 5 and 6, while increasing salt concentration clearly led to decreases in the adsorption effectiveness of impurities that caused to decrease in the purification percentage. The maximum purification percentage for these two types of solution which mentioned above is shown in Figure 7. The reason of decreasing this percentage linearly with $[NaCl]^{1/2}$ is that the adsorption of impurities decreases linearly with $[NaCl]^{1/2}$ in accordance with the predictions afforded by the Debye-Huckel theory (Hiemenz and Rajagopalan, 1997). This

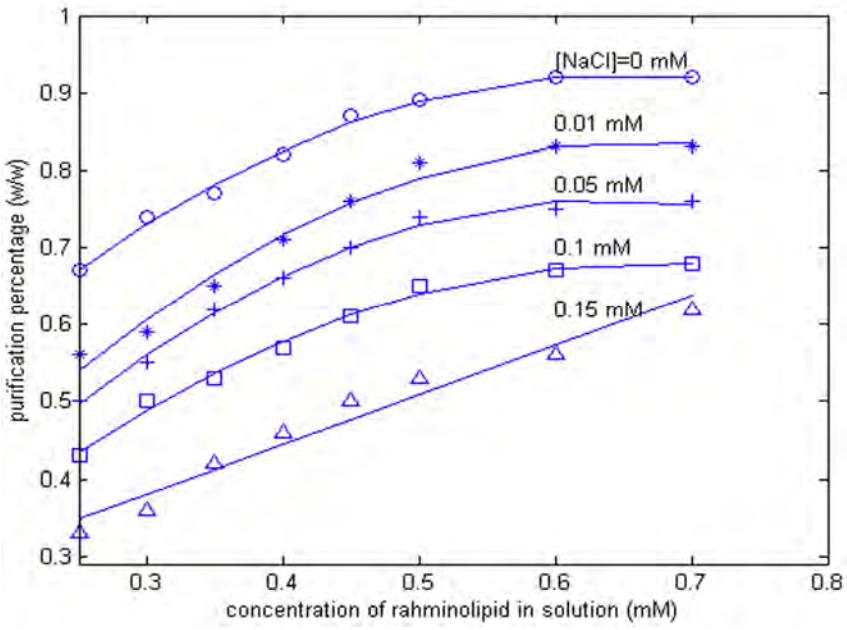

Figure 5. Effect of ionic strength on the rhaminolipid purification in MSM solution by DMPG - coated magnetic nanoparticles.

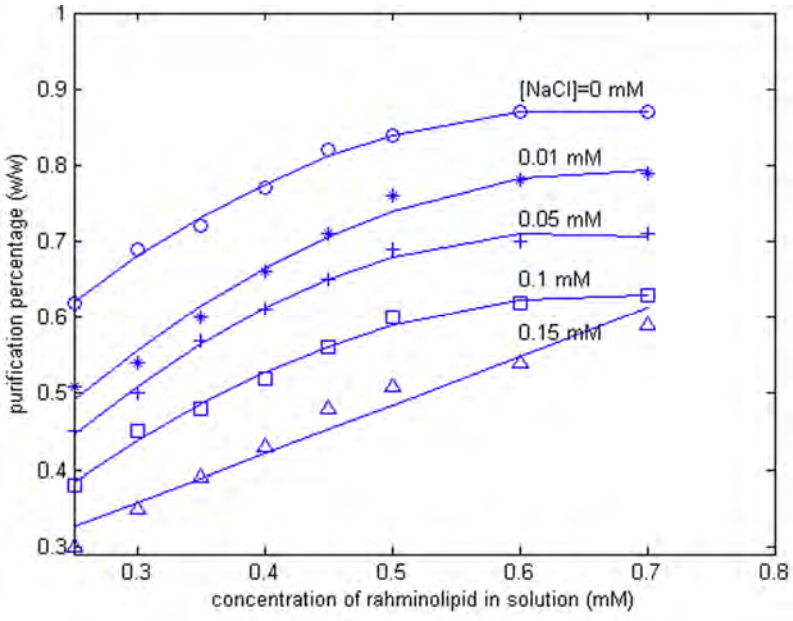

Figure 6. Effect of ionic strength on the rhaminolipid purification in MSM and strain solution by DMPG – coated magnetic nanoparticles.

behavior again points to the dominant role played by electrostatic interactions in this system. This observation was attributed to screening of the electrostatic interactions between the impurities and the charged phospholipid layers on the magnetic nanoparticles by salts, which reduced the adsorption of the impurities on the magnetic particles at neutral pH.

PH Effects

As shown in Figures 8 and 9, the purification has been increased by decreasing of pH. As observed, the purification ceased at pH 2 because by decreasing pH the solubility of rhaminolipid was decreased and amount of rhaminolipids precipitated. The net charge of impurities

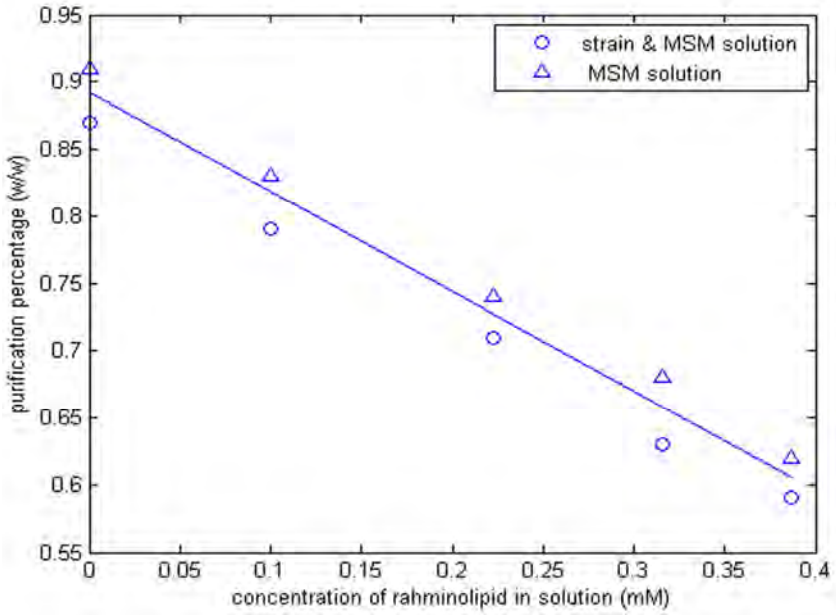

Figure 7. Variation in the maximum purification of Rhaminolipid and with [NaCl] 1/2. The linearity in Figure is in accord with the Debye - Huckel theory for screening of electrostatic interactions.

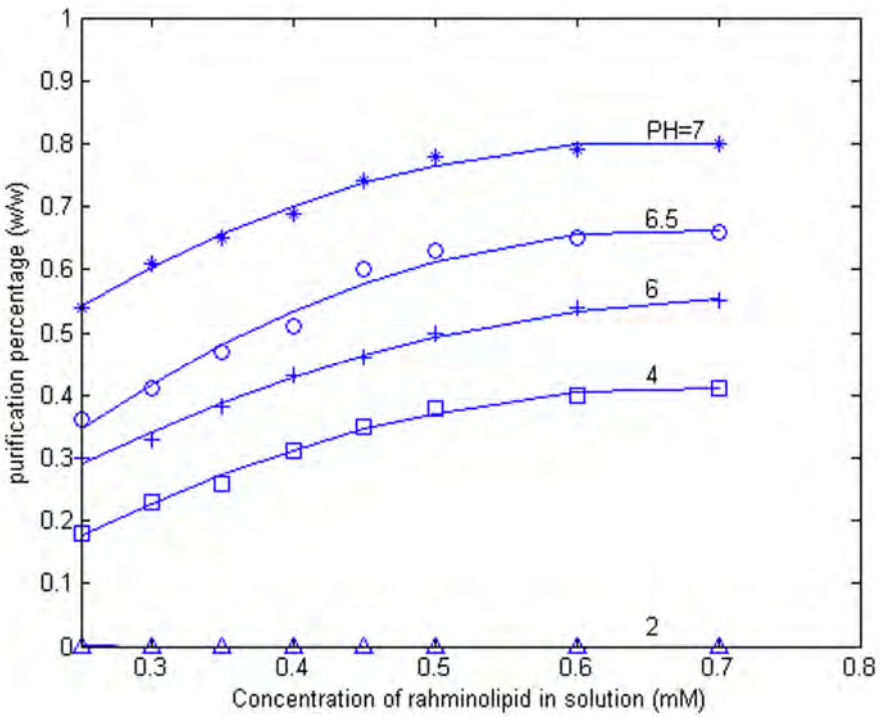

Figure 8. Effect of solution pH on Rhaminolipid purification in MSM solution by DMPG - coated magnetic nanoparticles.

was decreased with decreasing of pH which it led to reduce the interaction between impurities and phospholipid surface of nanoparticles.

Conclusion

There is continuing scope for the development of

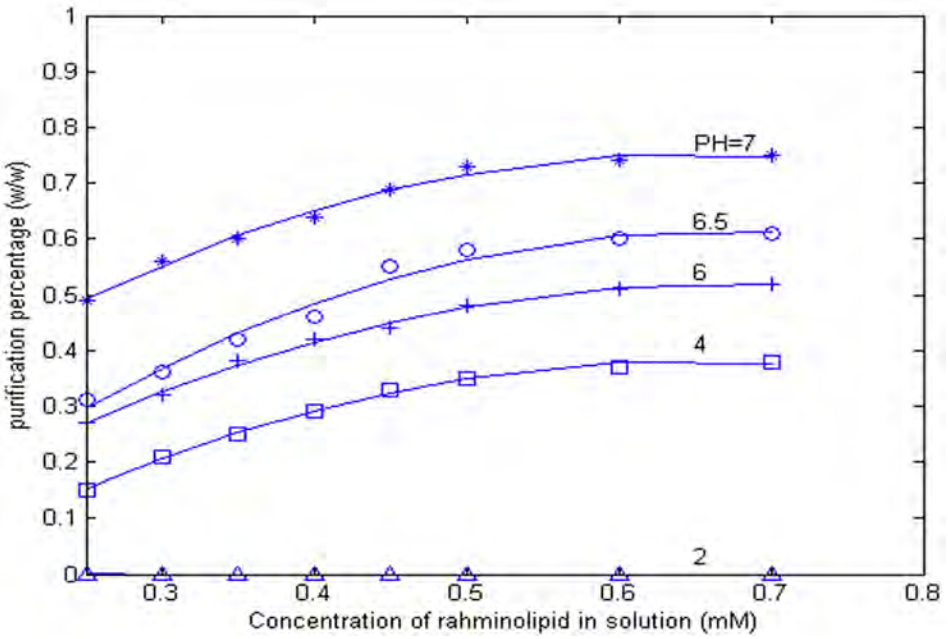

Figure 9. Effect of solution pH on Rhaminolipid purification in MSM solution by DMPG-coated magnetic nanoparticles.

enhanced separations and purification capabilities in the bio-processing industries. This need is particularly evident for technologies that provide high capacity and selectivity coupled with minimal diffusion limitations for macro-solutes, while also accommodating significant colloidal contaminants that would normally clog chromatography columns or foul membrane filters. To this end, we have explored the use of tailored magnetic nanoparticles to separate the strains and MSM mixtures.

The magnetic nanoparticles were coated with an inner chemisorbed fatty acid layer on which an outer phospholipids layer was adsorbed. The phospholipids shell provided both the interaction sites for the charge-based impurities adsorption and for the electrostatic stabilization of the magnetic nanoparticle suspension.such as strains loadings of approximately 1200 mg/ml adsorbent were obtained, several-fold higher than possible with the best commercially available chromatographic beads, which can be attributed to the size and dispersion characteristics of the magnetic nanoparticles. In the broader context, magnetic nanoparticles combined with strong magnetic field provide a potentially effective means for the rapid recovery and purification not only of macro solutes from suspensions of other colloidal materials but also of a wide range of other solutes, including environmental contami-nants (Moeser et al., 2002; Rashedi et al., 2006).

The flexibility afforded by the synthesis procedure allows for a range of functionalities to be incorporated on the particle surface and provides a versatile strategy for developing new tools for a wide range of chemical processing applications.

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