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ORIGINAL ARTICLE

Enhanced biodegradation of phenol by magnetically immobilized *Trichosporon cutaneum*

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Abstract

Aromatic compounds are abundant in aqueous environments due to natural resources or different manufacturer's wastewaters. In this study, phenol degradation by the yeast, *Trichosporon cutaneum* ADH8 was compared in three forms namely: free cells, nonmagnetic immobilized cells (non-MICs), and magnetically immobilized cells (MICs). In addition, three different common immobilization supports (alginate, agar, and polyurethane foams) were used for cell stabilization in both non-MICs and MICs and the efficiency of phenol degradation using free yeast cells, non-MICs, and MICs for ten consecutive cycles were studied. In this study, MICs on alginate beads by 12 g/l Fe_2O_3 magnetic nanoparticles had the best efficiency in phenol degradation (82.49%) and this amount in the seventh cycle of degradation increased to 95.65% which was the highest degradation level. Then, the effect of magnetic and nonmagnetic immobilization on increasing the stability of the cells to alkaline, acidic, and saline conditions was investigated. Based on the results, MICs and non-MICs retained their capability of phenol degradation in high salinity (15 g/l) and acidity (pH 5) conditions which indicating the high stability of immobilized cells to those conditions. These results support the effectiveness of magnetic immobilized biocatalysts and propose a promising method for improving the performance of biocatalysts and its reuse ability in the degradation of phenol and other toxic compounds. Moreover, increasing the resistance of biocatalysts to extreme conditions significantly reduces costs of the bioremediation process.

Keywords Alginate · Biodegradation · Magnetic and nonmagnetic immobilization · Nonmagnetic immobilization · Phenol · Polyurethane · *Trichosporon cutaneum*

Introduction

Phenolic compounds are one of the ubiquitous aromatic pollutants in the environment that naturally secret from plant roots or produce as a degradation intermediate of some complicated aromatic compounds such as lignin, naphthalene, anthracene, and other aromatic amino acid precursors (Krastanov et al. 2013; Rosenkranz et al. 2013). In addition, phenol is produced as a by-product by many industrial processes such as dyes, plastics, drugs, and petrochemical industries, and due to its high water solubility and toxicity, it could be a serious problem in the water and groundwater (Rosenkranz et al. 2013; Arora and Bae 2014). A lot of research has been done during the past years to remove micropollutants from wastewaters (Khansary et al. 2017; Masoudi et al. 2017; Moghimi et al. 2017). Different physical, chemical, and biological methods have been used for the degradation of phenolic compounds but due to their cost-effectiveness, ability to complete mineralization, and low possibility of by-product formation, biological methods are commonly preferred (Rocha et al. 2007). In previous studies, bacterial strains have gained more attention, but there are some yeasts genera such as *Trichosporon*, *Rhodotorula*, and *Candida* that can metabolize phenolic compounds as a source of carbon and energy (Alexieva et al. 2008). *Trichosporon cutaneum* is distinguished as one of the most widely used aromatic compounds degrading fungus (Alexieva et al. 2008; Yotova et al. 2009). In recent years, many attempts such as microbial cell immobilization, magnetic activation of free cells, and using adsorbent nanoparticle have been made in order to increase the affectivity and stability

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of microorganisms so as to degrade different contaminants (Wang et al. 2007; Zhang et al. 2011; Jiang et al. 2015).

The toxicity of phenolic compounds, especially at high concentrations, causes the washout of many microbial cells in the continuous batch and prevents the continuation of the degradation process. For this reason, immobilization of phenol degrading microbial cells could help to increase the efficiency of remediation (Chen et al. 2002). In addition, immobilized cells had more degradation activity and tolerance to the highly toxic phenolic compounds (Ailijiang et al. 2016). On the other hand, mass transfer limitation for substrate diffusion is the main problem that can reduce the degradation efficiency of immobilized cells with entrapment techniques (Jiang et al. 2015). Nanoparticles have been used as an adsorbent or a part of immobilization process in the function of an effective method to solve the mass transfer limitation of immobilized cells. Large specific surface and high surface activity in nanoparticles are responsible for this feature (Wang et al. 2007; Zhang et al. 2011).

The use of magnetic nanoparticles (Fe_2O_3), as a part of immobilization support for increasing the rate of mass transfer, was broadly used in various fields such as drug delivery, biosensing, and bioremediation process (Hou et al. 2016). This technique is considered as a solution for mass transfer resistance and facilitates the recovery of biocatalysts (Hou et al. 2016). For example, Zhang et al. (2010) reported that the usage of Al_2O_3 nanoparticles as adsorbent could increase the rate of biodesulfurization. In addition, magnetic immobilization by Fe_2O_3 nanoparticle could help in cell recovery and reuse (Zhang et al. 2011).

In this study, the stability and immobilization efficacy of *T. cutaneum* in different cell supports were compared in two forms of Fe_2O_3 magnetic nanoparticle-coated cells and non-coated cells for improving phenol degradation. In addition, the stability of non-magnetically immobilized cells (non-MICS) and magnetically immobilized (MICS) cells in different pH, salt, and biocatalysts reuse were studied.

Materials and methods

Chemicals

Glucose, phenol, peptone, yeast extract, and salts for Bushnell Haas medium were obtained from Merck (Germany). Agar, sodium alginate, polyurethane foam, Fe_2O_3 nanoparticles and Folin-Ciocalteu were obtained from Sigma Aldrich (USA). All chemicals used were of high purity.

Microorganism and cultivation

The yeast strain *T. cutaneum* ADH8 had previously been isolated from crude oil-contaminated soil and the high capability of this strain in phenol degradation had been proven (unpublished data). The identification of this strain was done by the amplification of ITS gene with the two ITS1 and ITS4 primers, and its sequence data were deposited in the National Center for Biotechnology Information (NCBI) databases under the KT962836.1 accession number. This strain was stored on glucose yeast peptone (GYP) plates at 4 °C. To prepare fresh preculture, 50-ml Erlenmeyer flasks containing 10 ml of Bushnell–Haas minimal medium supplemented with 1% (*w*/*v*) glucose were used (all aqueous cultures were incubated on a rotary shaker at 120 rpm and 28 °C). After reaching the absorbance of cultures to 0.6 at 600 nm, the yeast cells were harvested by centrifugation at 4000 rpm for 10 min. Then, the pellets were washed two times with normal saline and resuspended in an equal volume of normal saline and prepared for inoculation in different flasks (Hasanizadeh et al. 2017).

Fe_2O_3 nanoparticles coating on the yeast cells

To investigate the appropriate amount of Fe_2O_3 nanoparticles, different concentrations of nanoparticles (3–21 g/l) were used and the effect of different concentration and the optimal amount of nanoparticles were determined (Wang et al. 2007). To prepare the magnetically immobilized cells stab, Fe_2O_3 nanoparticles in different concentrations were added to 10 ml of the final cell suspension prepared as described in previous sections and shaken for 24 h at 28 °C and 120 rpm. After that magnetic nanoparticles and coated yeast cells were separated from the supernatant by a magnet and the remaining solution was removed, then the coated yeast cells were washed four times with 4 ml phosphate buffer and resuspended with 10 ml sterilized normal saline for immobilization.

Preparation of gel beads for immobilization of magnetically and non-magnetically nanoparticles coated yeast cells

According to the previous section, *T. cutaneum* ADH8 preculture cells were prepared and both yeast cells coated with Fe_2O_3 nanoparticles and free suspension cells were immobilized on agar beads, alginate beads, and polyurethane foam. Finally, six various immobilized yeast cells were prepared including non-MICs and MICs on alginate beads, agar beads, and polyurethane foam.

Agar entrapment of yeast cells

The method described by López and Lázaro NandMarqués (1997) was used to entrap cells in agar beads. Two grams of agar was dissolved in 90 ml normal saline solution at 100 °C and sterilized by autoclaving. After cooling to 50 °C, 10 ml of the cell suspension was added to agar solution and completely mixed. In order to the formation of the agar beads, the

suspension of agar and cells was added through a sterile syringe into normal saline. After stirring for 15 min, it was allowed to solidify for 2 h in the aqueous phase. Then, agar beads were washed twice with normal saline and were used for inoculation in the flasks containing phenol for degradation evaluation.

Alginate entrapment of yeast cells

For alginate beads entrapment, the method of Woodward (1988) was used. Four grams of sodium alginate was dissolved in 90 ml normal saline solution and then was sterilized by autoclaving. After cooling to 55 °C, 10 ml of the preculture cell suspension was added to sodium alginate solution and was completely mixed. Immobilized cells in alginate beads were formed by extruding the mixture through a 10-ml sterile syringe into 0.2 M $CaCl_2$ and letting it solidify for about 2 h. The alginate beads were washed two times with normal saline and were used for inoculation in the flasks containing phenol.

Yeast cell immobilization on polyurethane foams

The polyurethane foams were prepared by the method of Manohar et al. (2001) and were used for the immobilization of yeast cells. The polyurethane foam was cut into 5-mm cubes, washed twice with distilled water and left to dry. One hundred milliliters preculture medium containing fresh yeast strain was added to 500-ml flasks containing 4 g of sterilized polyurethane cubes and was stirred for 2 h by a magnetic stirrer. Then, the flask was placed on a rotary shaker at 120 rpm for 1 h and then remained constant for 2 h. The medium around the cubes was discarded, and the polyurethane cubes harboring the immobilized cells were washed twice with sterile normal saline and were used for inoculation into the flasks containing phenol for degradation rate assay.

Evaluating the efficiency of immobilization process and optimization

To obtain the number of immobilized cells in each immobilization support and doing equal inoculation between free and immobilized cells, a technique described by Liao and Chen (2001) was used. The number of un-immobilized cells in $CaCl_2$ solution for alginate beads, normal saline for agar beads and medium containing polyurethane foam was calculated. Five milliliters of $CaCl_2$ solution and normal saline for alginate beads and agar beads, respectively and 1 ml of medium containing polyurethane foam pour plated in appropriate GYP plates were incubated at 28 °C for 48 h. After that, the number of colonies was counted, multiplied by the dilution factor and an approximate number of un-immobilized cells was calculated to determine the appropriate amount of inoculum, it was necessary to demonstrate the number of immobilized cells, so the number of un-immobilized cells was counted and after that, the number of un-immobilized cells was subtracted from an initial number of cells and the number of immobilized cells was determined. Finally, it was revealed that for the approximately equal inoculation of immobilized cells and free cells, the amount of inoculation should be 2 ml for agar beads and alginate beads and 0.438 g of polyurethane foam in each flask. This test was performed in three replicates and the values obtained for inoculation were equal to the average of these three replicate results.

Evaluation of phenol degradation by free and immobilized cells

Biodegradation experiments were done in 50-ml flasks containing 9 ml Bushnell–Haas for free cells and 10 ml for beads; all flasks were supplemented by 1500 mg/l phenol as a sole carbon source. Ten percent inoculation (1 ml of pre-cultures) was used for free cells and 2 ml of the initial suspension in the preparation of alginate and agar beads and 0.44 g of polyurethane foam for immobilized cells were used. After 24 and 48 h, the phenol concentration in each culture was determined (Wang et al. 2007; Zhou et al. 2011). In this experiment, immobilized and magnetically immobilized inactive yeast cells were used as a control and all cultures were performed in three replicates. Inactivation of yeast cells in control samples was done by autoclaving at 121.5 °C and 15 psi/l for 20 min.

For evaluation of reused cells efficiency, the free and immobilized cells were collected after 48 h and were washed twice with normal saline, then 10 ml of the medium with 1500 ppm phenol was added to repeat the cycles (up to 10) (Jiang et al. 2015).

pH and salinity stability

The influence of pH (5 to 8) and the presence of NaCl (up to 15% (*w*/*v*)) on the phenol degradation by *T. cutaneum* ADH8, in the three circumstances (free, non-MICs, and MICs) were examined. For this purpose, similar to the previous steps Bushnell–Haas medium containing 1500 ppm phenol with different concentrations of NaCl (0, 2.5, 5, 7.5, 10, and 15% (*w*/*v*)) and pH 5, 6, 7, and 8 were prepared. The condition and amount of inoculation were completely the same as earlier stated. After inoculation, cultures were incubated at 120 rpm and 28 °C in three replicates and the density of phenol was calculated every 24 h.

Measuring phenol concentration

To measure the concentration of phenol, the Folin-Ciocalteu method was used (Senthilvelan et al. 2014). In

each sampling, 1 ml of the cultures was taken and centrifuged at 10,000 rpm for 10 min, and then 0.3 ml of the samples was added to 1.2 ml of 20% (*w*/*v*) sodium carbonate and was incubated at room temperature for 8 min. Thereafter, 1 ml of Folin-Ciocalteu was added to it and then incubated for 40 min at 56 °C, and then the absorbance of the samples was measured at 750 nm. The absorbance of the samples was compared with standard curve, and the phenol density in each culture was determined (Singleton et al. 1999; Senthilvelan et al. 2014).

Statistical analysis

The experimental results of this paper were treated statistically using SPSS version 24. The Kolmogorov-Smirnov test was used to assess the normality of distribution of investigated parameters. All data in this study were distributed normally. Data were expressed as mean ± standard deviation. Differences were tested by chi-square test. Pearson's correlation was used to analyze the association between all studied parameters. The values $P < 0.05$ were considered statistically significant.

Results and discussion

Optimization of Fe_2O_3 nanoparticle concentration

The effect of different concentrations of Fe_2O_3 nanoparticles in the phenol degradation by MICs in alginate beads was investigated. The results are as shown in Fig. 1 ($P < 0.05$). Non-MICs and free cells degraded about 927/33 mg/l (61.82%) and 1054.76 mg/l (70.32%) of phenol in 48 h. Figure 1 shows that using 12 g/l of Fe_2O_3 can reach the highest rate of phenol degradation in 48 h. Increasing the rate of phenol degradation in the presence of Fe_2O_3 magnetic nanoparticles as part of immobilization support shows the role of magnetic nanoparticles in the reduction of mass transfer resistance and an acceleration in material transport. Moreover, a high concentration of nanoparticles (18 and 21 g/l) had an inhibitory effect on the efficiency of phenol degradation due to filling the pores of immobilization support (Jiang et al. 2015).

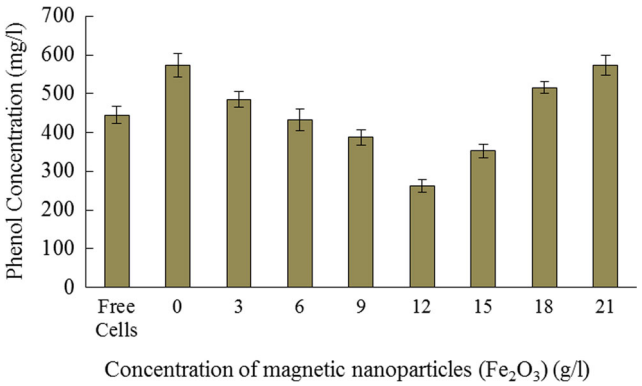

Fig. 1 The amount of remained phenol at the end of 48 h in different concentrations of Fe_2O_3 nanoparticles

The use of magnetic nanoparticles such as Fe_2O_3 and Al_2O_3 as part of immobilization support, can help in more easily and quickly collection of biocatalysts (Zhang et al. 2011). When the concentration of Fe_2O_3 nanoparticles was 6 g/l or lower, it was not possible to separate the magnetic beads from nonmagnetic beads by a magnet but at 9 g/l or concentration higher than 9 g/l, magnetic beads were easily separated from nonmagnetic beads just by a magnet. In similar studies, the appropriate concentration of Fe_2O_3 nanoparticles for magnetic immobilization process has been reported and it showed various concentrations such as 9 g/l for magnetic immobilization of *Rhodococcus rhodochrous* for degradation of chlorophenols (Hou et al. 2016) and even 100 to120 mg/l for magnetic immobilization of *Comamonas* sp. for degradation of BTEX-containing wastewaters (Jiang et al. 2015). In addition, in a different study, Zhang et al. (2011) investigated the ratio of cell weights to nanoparticles and showed that when this ratio is 200:1 (g/g), the best efficiency of desulfurization has occurred and also they have shown that when this ratio is 50:1 (g/g) or more, magnetic separation of magnetic cells from nonmagnetic cells is possible.

Phenol degradation by free and immobilized *T. cutaneum* cells

The obtained results from phenol degradation by *T. cutaneum* ADH8, in three different conditions including free, MI, and non-MI cells are shown in Table 1 ($P < 0.05$). According to the results, the rate of phenol degradation by non-MICs compared to free cells (70.32%) has been decreased to 61.82, 56.94, and 60.02% for alginate beads, agar beads, and

Table 1 The rates of phenol degradation by free, non-MI, and MI cells

Types of cells	Phenol degradation (%)	
	After 24 h	After 48 h
Free cells	20.42	70.32
Non-MICs on alginate beads	15.31	61.82
MICs on alginate beads	27.15	83.82
Non-MICs on polyurethane foam	11.97	60.02
MICs on polyurethane foam	22.38	80.41
Non-MICs on agar beads	9.44	56.94
MICs on agar beads	17.12	67.51

Non-MICs non-magnetically immobilized cells, *MICs* magnetically immobilized cells

polyurethane foam, respectively. Nevertheless, with the use of magnetic immobilization, not only the rate of phenol degradation increased as compared to non-MICs but also the rate of phenol degradation for polyurethane foam and alginate beads has been more than that of free cells and were 80.41 and 83.82%, respectively. Also, the rate of phenol degradation by immobilized cells on magnetic agar beads as compared to nonmagnetic agar beads increased 10%, but was still less than the efficiency of free cells.

These results clearly showed the positive effect of using Fe_2O_3 nanoparticles and magnetic immobilization on increasing the efficiency of phenol degradation. In order to remove the effect of iron nanoparticles as an absorbent and demonstrate the magnetic effect of nanoparticles on reducing mass transfer resistance, immobilized inactive cells by magnetic method were used as control. In a similar study, Wang et al. (2007) demonstrated that the rate of carbazole degradation can be improved by magnetic immobilization, and they showed that free cells consume 3.340 μg of carbazole in 20 h, but non-MICs and MICs consume the same amount of carbazole in 36 and 16 to18 h, respectively. Also, Ebrahiminezhad et al. (2016) and Raee et al. (2018) announced that by magnetic immobilization of *Bacillus subtilis natto* and *Escherichia coli*, their capability in producing menaquinone-7 and asparaginase were increased, respectively about 15 and 26%.

Effect of biocatalysts reuse in phenol degradation

In biological processes that are carried out on the industrial scale, recycling of biocatalysts and their performance after each cycle is considered as an important factor in determining the effectiveness of biocatalysts (Jiang et al. 2015). The efficiency of phenol degradation by MICs and non-MICs was tested repeatedly up to ten cycles and the results are as shown in Fig. 2 ($P < 0.01$). As shown in Fig. 2, the phenol degradation by reusing immobilized cells on agar beads decreased faster than alginate beads and polyurethane foam. Furthermore, agar beads from the fifth cycle began to fracture which shows that the use of agar beads as immobilization supports is not suitable for continuous processes.

Alginate beads and polyurethane foams had a good strength and during ten consecutive cycles, they did not have any fracture or breakdown. Non-MICs on alginate beads and polyurethane foam from the first to the fifth cycle and MICs on alginate beads and polyurethane foam from the first to the seventh cycle increased in phenol degradation. However, phenol degradation reduced generally in these biocatalysts from the fifth to the tenth and seventh to the tenth cycle, respectively. Agar beads were much more fragile than alginate beads and polyurethane foams, and therefore had the least rate of phenol degradation during the ten continuous cycles. In comparison of alginate beads and polyurethane foams, it can be said that the

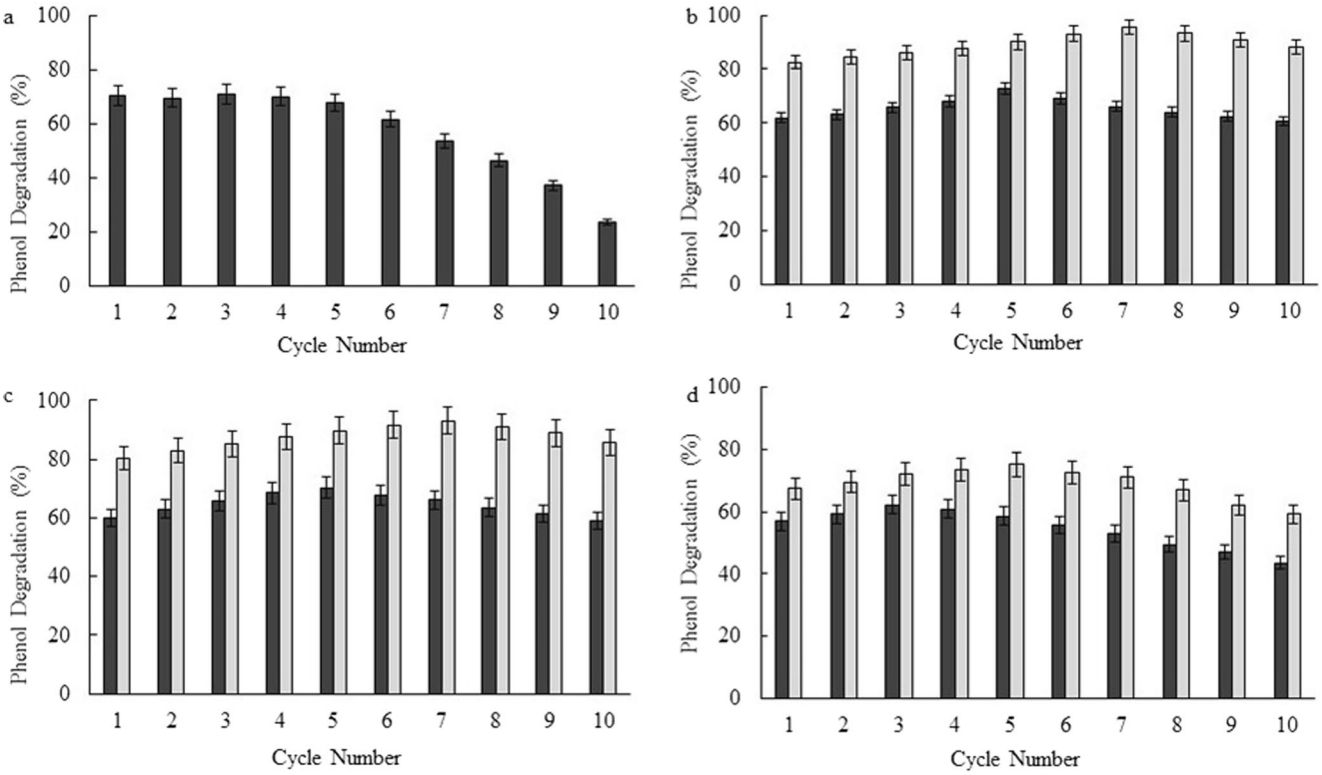

Fig. 2 **a** Rae of phenol degradation in continues batches by free cells. **b** Alginate beads. **c** Polyurethane foam. **d** Agar beads. Magnetically immobilized cells (white column) and nonmagnetic immobilized cells (black column)

yeast cells were encapsulated in the alginate beads and immobilized on the surface of polyurethane foam; encapsulation of yeast cells in the alginate beads probably was the factor contributing to the stability of these cells in continuous cycles. According to the results, MI cells on alginate beads had the best efficiency of phenol degradation during consecutive batches and in the seventh cycle for MICs on alginate beads, phenol degradation increased to 95.65 from 82.49% in the first cycle. The results revealed that by using an appropriate concentration of Fe_2O_3 nanoparticles due to magnetic immobilization of yeast cells, the efficiency of phenol degradation during consecutive cycles can be increased (Fig. 2). In previous related studies, Hou et al. (2016) demonstrated that the magnetic immobilized *Rhodococcus rhodochrous* can strongly degrade chlorophenols from the first to the sixth cycle. Also, Wang et al. (2007) declared that when MICs are reused from the first cycle to the fifth cycle, the rate of carbazole degradation stayed practically constant and from the fifth to the eighth cycle, the degradation rates were increased and in the contrast Shi et al. (2014) announced that after the stability of degradation rate by MICs in the first to the fifth cycle and from the sixth to the seventh cycle, the degradation rates slightly decreased.

Effect of immobilization on pH and salt (NaCl) stability

The use of microorganisms that can degrade various pollutants in the presence of NaCl and abnormal pH can reduce the cost of the bioremediation process (Bastos et al. 2000). Many areas with phenolic contaminations, such as petrochemical and crude oil refinery wastewaters have different concentrations of salt and therefore for direct use of biocatalysts, halotolerant and halophile microorganisms should be used. Many attempts have been made to find phenol degrading microorganisms that are capable of proper growth in the presence of salt, among which are *Candida tropicalis*, *Alcaligenes faecalis*, and *Halobacterium halobium* (Bastos et al. 2000; Peyton et al. 2002).

As shown in Fig. 3 when the NaCl concentration was 15% (*w*/*v*), the rate of phenol degradation by free cells reduced to 25% of the initial phenol degradation rate ($P < 0.01$), but when non-MICs and MICs were used, phenol degradation rate at 15 g/l NaCl, decreased to 58 and 84% of the initial degradation, respectively ($P < 0.05$). Although the rate of phenol degradation by *T. cutaneum* ADH8 at the presence of 15% (*w*/*v*) NaCl was lower than the amount of phenol degradation by halophile and halotolerant microorganisms indicated in other studies (Bastos et al. 2000; Peyton et al. 2002), but phenol degradation by MICs was greater. This result supports the idea that magnetic immobilization can be a suitable choice for improving the function of microorganisms in saline wastewater and environments.

As shown in Fig. 3, the efficiency of phenol degradation by free cells in pH 5 and 8 was reduced to 29 and 13%, respectively ($P < 0.01$). However, when an immobilized cell was used at pH 5, about 90 and 97.5% of the phenol degradation ability

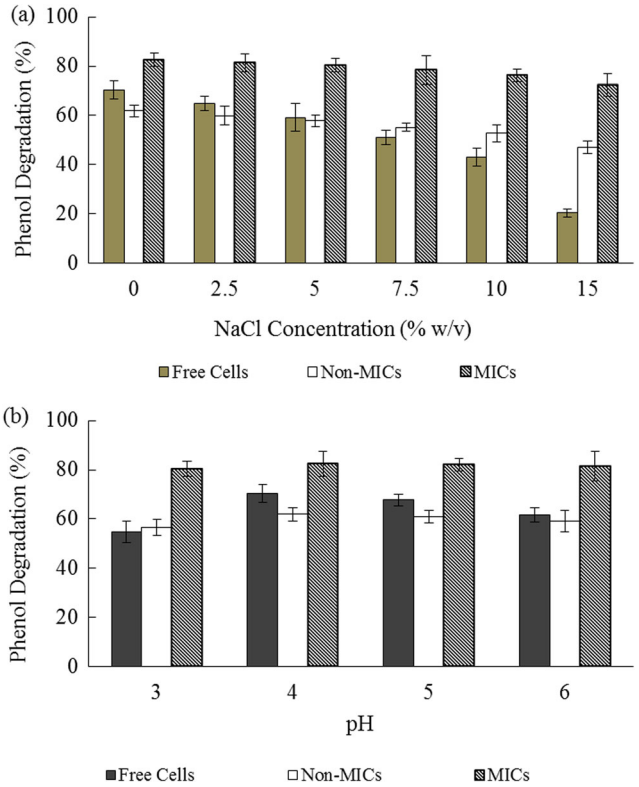

Fig. 3 The rate of phenol degradation at the end of 48 h, **a** in different NaCl concentration and **b** in different pH

for non-MICs ($P < 0.05$) and MICs remained (Fig. 3). The results showed that *T. cutaneum* generally had a better degradation in alkaline pH rather than acidic pH, but still at pH 8 just 3% of non-MICs phenol degradation efficiency reduced and the rate of phenol degradation by MICs was approximately equal to pH 6. Another study in 2016 showed that by immobilization of *Acenitobacter* sp., its production of 3-hydroxypropionic at pH 3 would be increased from about 55 to 85% (Jiang et al. 2013).

On the other hand, analysis of the results of pH and NaCl changes on the efficiency of phenol degradation by MICs showed that these changes are not statistically significant ($P > 0.05$) and can be said that pH and NaCl changes are ineffective (between 5 and 8) on the efficiency of MICs. By intracellular immobilization of yeast cells in the alginate beads, the stability of cells against the salinity and acidity has increased and it could be because when yeast cells are encapsulated in alginate beads a small interior space is created and separated them from the outer space, which allows the cells to control the conditions by secreting less amount of different compounds.

Conclusion

This study investigated the consequences of magnetic and non-magnetic immobilization on phenol degradation activity of *T.*

cutaneum in different conditions. The results showed that magnetic immobilization can properly increase the phenol biodegradation efficiency in optimal circumstances and special conditions such as acidic and alkaline pH or the presence of saline. It was also confirmed that using 12 g/l of Fe_2O_3 for magnetic immobilization is the optimal concentration for degradation activity. The comparative study of different immobilization supports showed that alginate beads and polyurethane foams are much better than agar beads in resistance to breakage, stability in consecutive processes, and phenol degradation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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