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Biodegradation of high concentrations of phenol by baker's yeast in anaerobic sequencing batch reactor

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Abstract

Background: Phenol, as a pure substance, is used in many fields because of its disinfectant, germicidal, local anesthetic, and peptizing properties. Aqueous solutions of phenol are produced as waste in industries and discharged into the environment. Therefore, elevated concentrations of phenol may be found in air or water because of industrial discharge or the use of phenolic products.

Method: The strains of *Saccharomyces cerevisiae* used in this project were natural strains previously purchased from Razavy company. They were grown at 30°C on Petri plates containing yeast extract glucose (YGC) and then purified by being spread onto new plates, and isolated colonies were obtained. These colonies provided the basis of selection. Prepared strains were applied in anaerobic sequencing batch reactors (ASBRs) as first seed. The experiment conditions were optimized using response surface methodology (RSM). After the determined runs were performed using Design-Expert software, data were analyzed using mentioned software as well.

Results: This study evaluated the capability of baker's yeast to remove phenol in high concentrations. The tested strains showed excellent tolerance to phenol toxicity at concentrations up to 6100 mg/L. Study of the batch degradation process showed that the phenol removal rate could exceed 99.9% in 24 hours at a concentration of 1000 mg/L. The results showed catechol is the first intermediate product of phenol degradation. In survey results of the Design–Expert software, R2 and Adeq precision were 0.97 and 25.65, respectively.

Conclusion: The results demonstrated that ASBR performs robustly under variable influent concentrations of inhibitory compounds. The high removal performance despite the high phenol concentration may be a result of reactor operating strategies. Based on the progressive increase of inlet phenol concentration, allowing for an enhanced biomass acclimation in a short time, results at the microbiological levels showed that the increase of phenol concentration was accompanied by a decrease in the microbial community and a progressive selection of the most adapted phenotypes.

Keywords: Yeast, Phenol, ASBR, Biotransformation, Synthetic solutions, Design of experiments software

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Introduction

As a result of industrialization, mankind has encountered vast environmental concerns, despite improvements to the quality of life. Rapid accumulation of recalcitrant chemicals in the biosphere severely endangers the environment and human health (1). Phenol and its derivatives are released into the environment through wastewater discharges from industries such as oil refineries, coke ovens, coal conversion, the synthesis of chemicals, steel mills, pulp and paper, textile and pharmaceutical factories. Phenol concentrations in these effluents vary from 10 to 17500 mg/L.

Phenol has acute and chronic effects on human health. Inhalation and dermal exposure to phenol are considered acute effects (less than 14 days of exposure) and are highly irritating to skin, eyes, and mucous membranes. Other

acute health effects are headache, dizziness, fatigue, fainting, weakness, nausea, vomiting, and lack of appetite at high levels of phenol exposure. Effects from chronic exposure (longer than 365 days) include irritation of the gastrointestinal tract. Phenol can change blood pressure and cause liver and kidney damage, and the nervous system is also negatively affected by long-term exposure. The Environmental Protection Agency (EPA) has classified phenol as a group D, not classifiable as to human carcinogenicity. Animal studies have not shown tumors resulting from oral exposure to phenol, but dermal studies have reported that phenol applied to the skin may be a tumor promoter and/or a weak skin carcinogen in mice (2-4).

It is necessary to develop highly efficient techniques for the removal of these pollutants from wastewater to protect the environment. Several methods have been developed for phenol removal, such as precipitation, coagulation, ion exchange, and ultrafiltration; however, each of these methods suffers from one or more limitations (5). Biodegradation has been highly appreciated as the most versatile promising approach. Indeed, bioprocesses have been proven to be efficient approaches for the complete mineralization of organic compounds (6). Additionally, biological methods are preferred over physicochemical methods because of their economical aspects as well as nontoxic by-products. The anaerobic degradation of phenol offers the advantages of requiring no oxygen, producing low waste-biomass, and generating a valuable waste product - methane gas (7-10). In the available literature, no report on the treatment of phenol using an anaerobic sequencing batch reactor (ASBR) was found. Different anaerobic technologies have been implemented to cope with the presence of phenol because of the advantages they offer over other biological unit operations, eg., high organic loading rates, low sludge production, and energy production. Among them are ASBRs, given their various advantages such as operational simplicity, efficient quality control of the effluent, flexibility of use, and high biogas yield (4,11). Accordingly, understanding the various aspects of this process is essential to improve the efficiency of the system. Among microorganisms, yeasts have high capabilities in the degradation of recalcitrant compounds such as phenol. Thus, the primary objective of this study was to investigate the performance of the ASBR when treating a synthetic wastewater containing high concentrations of phenol.

Methods

2.1 Preparation of yeast

The *Saccharomyces cerevisiae* strains tested in this project are natural strains previously purchased from Razavy company. They were grown at 30°C on Petri plates containing yeast extract glucose (YGC) and then purified by spreading them onto new plates to obtain isolated colonies (Figure 1). These colonies provided the basis of selection.

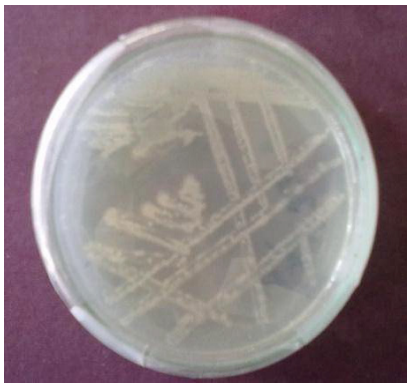

Figure 1. Image of the yeast culture in yeast extract glucose (YGC) medium under a temperature of 30°C.

Bioreactor and culture conditions

The optimal design of feeding is a very important aspect in developing the fermentation process. For the biodegradation studies feeding carried out in the bioreactor contained phenol at different concentrations in the range of (1000-6100 ppm) and was sterilized. Among the aromatic hydrocarbons, phenol is considered to be the principal component that inhibits cell growth (1), particularly when its concentration exceeds 500 mg/L. Moreover, high concentrations of phenol are converted to carbon dioxide at a lower rate than low concentrations (12). It has been reported that some strains of bacteria (6,13-15) or fungi (16,17), especially certain strains of yeast, are capable of converting phenol to carbon dioxide and water and are tolerant of phenol toxicity at concentrations of 500 mg/L or higher. A conventional ASBR to form high density polyethylene cylinders with a working volume of 4 L, an internal diameter of 15 cm, and a working height of 30 cm was operated under anaerobic conditions in 24-, 18.75-, 13.25-, 8.25-, and 3-hour cycles with nearly 95% of cycle time to reaction, about 2% of reaction time to feeding and withdrawal, and the rest to settling. The corresponding hydraulic retention time (HRT) varied between 3 hours and 24 hours depending on the cycle length. Solid retention time (SRT) was set at 10 h.

The initial biomass concentration was 0.01 g/L. Triplicate assays were carried out with 2 different types of carbon sources: only phenol and phenol + glucose. The initial phenol and glucose concentrations were 500 mg/L and 5 g/L, respectively. To avoid excess loss of sludge, the reactor was initially operated at the high settling time of 30 minutes. In the initial stage, feeding time was kept high was and then decreased gradually as the operation proceeded. The reactor was fed with synthetic wastewater containing phenol at different concentrations and sodium bicarbonate to maintain alkalinity in the range requirement.

For microbial community characterization, samples were taken from the reactor at the end of the last operation cycle under each phenol concentration tested. The reaction time increased exponentially with inlet concentration, probably due to an inhibitory effect which required increasing the reaction period and operating the ASBR at a lower organic loading rate (OLR) (Figure 2).

Figure 2. Flow diagram of the anaerobic sequencing batch reactor (ASBR) experimental setup.

Batch biodegradation assay

Optical density (OD) measurement is a common procedure usually used in predictive microbiology to follow microbial growth. It is easily applicable, rapid, and inexpensive compared with plate count methods. Growth curves were obtained based on OD at 600 nm, and the growth rates for the cells grown on each phenol concentration were simultaneously calculated. Cells were incubated at 37°C, centrifuge rate of 200 rpm, and pH of 7.5.

During 90 days of experiments, samples were collected at proper time intervals, centrifuged, and analyzed for pH, biomass, residual phenol, and glucose concentrations. Supernatants of the drawn samples were used for analysis.

Estimation of phenol concentration

To measure the residual phenol concentration, samples were centrifuged in a centrifuge for 5 minutes. Phenol concentration was detected by a direct photometric method based on the rapid condensation of 4-aminoantipyrene followed by oxidation with alkaline potassium ferricyanide and resulting in red coloration which can be measured by a spectrophotometer UV-VIS +T80. The developed procedure was in accordance with standard methods (18).

Estimation of biomass concentration

The biomass concentration was estimated using the dry weight method. A 50 ml sample of culture broth was withdrawn from the bioreactor and centrifuged at 4000 rpm for 5 minutes in plastic centrifuge tubes. The supernatant was decanted into small bottles and stored at 40°C for subsequent phenol estimation. The pellets were rinsed off the tube into a preweighed 1.2 millipore filter paper (Whatman GF/C). The filter paper was then dried in an oven at 105°C for 12- 24 hours, cooled in a desiccator at room temperature, and reweighed until a constant dry weight was obtained. The difference between the preweighed filter paper and the second weight was used to estimate the dry weight of the biomass (19).

Measurement of catechol

To obtain the chromatogram of catechol, the concentration of the phenolic compound was determined by high performance liquid chromatography (HPLC). The gradient elution of 2 solvents was used – mobile phase solvent A (20 mM phosphoric acid [pH 2.0 unadjusted]) and solvent B (acetonitrile) (75:25, A:B). Column Ascentis® RP-Amide 15 cm × 4.6 mm I.D., 5 µm particles (565324-U) was used. Flow rate was 1.5 ml/min. The temperatures of the injector and detector were fixed at 30°C and the sample was injected at a rate of 25 µl. The biotransformation compounds formed by phenol were monitored by the detector UV, 270 nm.

Design of experimental methodology

A design of experiments was applied using Design-Expert 6.0 (trial version). Design study was performed with 4 variables at 2 levels to explore the effects of variables on the response in the investigated region. Biomass concentration, phenol concentration, HRT, and pH were considered the most effective independent variables. A total of 30 experiments with 4 variables was performed and augmented with 8 replications at the center points to evaluate the pure error. The experiments were conducted in an ASBR reactor as described in the Methods section. Percentage of phenol removed was taken as the response. Experiments were performed according to the experimental design matrix given in Table 1 and within the ranges indicated.

The matrix for five variables was varied at 5 levels ($-\alpha$, −1, +1, $+\alpha$). The higher level of variable was designed as "+1," the lower level was designed as "−1," and star points were designed as "$-\alpha$ and $+\alpha$." In the optimization process the response can be related to the chosen variables by linear or quadratic models (Table 1).

Results

The results of model statistical analysis

In this part explanations of the study results obtained by analysis design of experimental software are presented (Table 2).

Table 1. Points selected for RSM design

Parameter	Code	-1 Level	+1 Level	- Alpha	+ Alpha
Phenol concentration	A	2275	4825	1000	6100
pH	B	4.38	7.125	3	8.5
Biomass concentration	C	0.50	1.50	0.01	2
HRT	D	8.25	18.75	3	24

Abbreviations: RSM, response surface methodology; HRT, hydraulic retention time.

Table 2. Results of ANOVA for response surface reduced quadratic model

Source	Sum of squares (SS)	Degrees of freedom (DF)	Mean square (MS)	F value	P value, Prob > F
Model	0.061	14	4.348	40.48	<0.0001
A	0.053	1	0.053	493.55	<0.0001
B	3.504	1	3.504	32.62	<0.0001
C	5.042	1	5.042	4.69	0.046
D	3.750	1	3.750	0.35	0.563
AB	1.562	1	1.562	1.45	0.246
AC	3.062	1	3.062	2.85	0.112
AD	5.625	1	5.625	0.52	0.480
BC	1.056	1	1.056	9.83	0.006
BD	3.063	1	3.063	2.85	0.112
CD	1.563	1	1.563	1.45	0.246
A2	8.048	1	8.048	7.49	0.015
B2	6.519	1	6.519	0.61	0.448
C2	2.333	1	2.333	0.022	0.884
D2	1.174	1	1.174	10.93	0.0048
Residual	1.611	15	1.074		
Lack of fit	1.011	10	1.011	0.84	0.619
Pure error	6.000	5	1.200		
Core total	0.062	29			

Mean = 0.91; SD = 0.010; R-squared = 0.97; adjusted R-squared= 0.95.

Optimization of reaction conditions with response surface methodology

As mentioned in the Methods section, the pH and phenol concentrations were crucial parameters together with biomass concentrations and HRT. Design study was performed based on these parameters and obtained results are given in Table 3.

As a result of the analysis, the following model for coded factors was obtained (Equation 1).

|% Removal = 0.92 - (0.047*A) - (0.012*B) + (4.583*C) - (1.250*D) - (3.125*A*B) - (4.375*A*C) - (1.875*A*D) - (8.125*B*C) + (4.375*B*D) + (3.125*C*D) - (5.417*A^2) - (1.542*$B^{2)}$) - (2.917*C^2) - (6.542*D^2)

The statistical optimization results were quite promising. Statistical analysis determined that approximately 92% removal was achieved under optimum conditions. According to experimental design method, pH, and phenol concentrations were found to be statistically significant because their *P* values were lower than 0.0001. The positive coefficient value of the biomass concentration indicated that it has a positive effect on phenol removal in contrast to phenol concentration which has a negative coefficient. The effects of phenol and biomass concentrations showed that the percentage of phenol removal increased with increasing biomass concentrations and decreased with increasing phenol concentrations. Time was found to be statistically non-significant because of a high *P* value. When the graph showing the interaction between the phenol concentration and contact time is analyzed; however, it can be seen that phenol removal shows a slight improvement between 3 hours and 24 hours as if it has an optimum value somewhere in-between.

The conditions of the optimum point predicted by the program can be seen in Table 4. In order to verify this prediction, confirmation experiments were performed in the same way as the optimization experiments (Table 5).

The results were between the confidence levels (Table 4) of the predicted percentage of phenol removal. This indicates that the model is useful within the given range of conditions, verifying the reliability of the model. The results clearly showed that parameters influence each other, and the ideal value for one depends on the values of others. This is a unique feature of the experimental design methodology compared with traditional methods in which the simultaneous observation of two parameters is very difficult.

The effects of considered parameters (pH, phenol concentration, HRT and biomass concentration) on phenol removal efficiency are given in Figures 3, 4, 5 and 6, respectively.

Optical density and growth measurement

A previous calibration curve was developed to obtain correlations between OD and growth time of yeast. Therefore, in this work, the area under the OD/time curve was considered an appropriate indicator of the overall yeast growth due to the fact that this value showed a clear proportionality (direct or inverse) with the kinetic growth parameters. As a graphic example, Figure 7 shows the plot of OD versus time for growing yeast under constant glucose

Table 3. Experimental design matrix and responses

Phenol concentration (ppm)	pH	Biomass concentration (g/l)	Hydraulic retention time (h)	Removal efficiency (%)
4825	4.38	0.51	18.75	85
3550	5.75	1.01	18.50	91
3550	8.50	1.01	13.50	90
1000	5.75	1.01	13.50	99.9
2275	4.38	0.51	18.75	94
2275	7.13	0.51	18.75	94
3550	5.75	1.01	13.50	92
4825	7.13	0.51	8.25	86
3550	5.75	2.00	13.50	93
4825	7.13	1.50	18.75	83
2275	4.38	1.50	18.75	97
3550	4.38	0.51	13.50	91
4825	7.13	0.51	18.75	85
3550	5.75	1.01	24.00	90
4825	7.13	1.50	8.25	83
3550	5.75	1.01	13.5	92
2275	7.13	1.01	18.75	95
3550	3.00	1.01	13.5	93
4825	4.38	0.51	8.25	88
3550	5.75	1.01	3.00	89
3550	5.75	1.01	13.5	92
2275	4.38	0.51	8.25	96
3550	5.75	1.01	13.5	94
4825	4.38	1.50	8.25	89
6100	5.75	1.01	13.5	80
4825	4.38	1.50	18.75	89
3550	5.75	1.01	13.5	91
2275	4.38	1.50	8.25	98
2275	7.13	0.51	8.25	93
2275	7.13	1.50	8.25	94

concentrations. Similar results were also obtained for the other yeasts (data not shown). In this way, the maximum area value was obtained when the slope of the growth curve and the upper OD asymptote were higher, but the lag phase was shorter.

Results of catechol measurement

The HPLC analysis of the culture medium with phenol showed a peak at 270 nm at 5.5 RT, which indicated that there was an intermediate of degradation of phenol compared with standard phenol, which showed a peak on 1 RT, which might indicate the formation of other intermediate compounds (Figure 8 and Table 6).

Discussion

Effect of pH

The medium pH is one of the significant factors in the success of the biological treatment process which significantly affects the biochemical reactions required for phenol degradation. Most of the microorganisms cannot tolerate pH levels above 9.5 or below 4.0. However, the optimum pH for different microorganisms is different. *S. cerevisiae* can adapt over a wide pH range, indicating that *S. cerevisiae* can be applied to different climates without the pH value changing. These results support the statement that the growth rate of most yeasts are usually maximum within the pH range of 4-5.5. Finally, the pH effects on phenol degradation were checked, because pH is a major factor controlling fungal growth. In the present work, the medium pH was considered to be 3-8.5 for further degradation studies. As illustrated in Figure 3, the rate of phenol degradation by *S. cerevisiae* did not fluctuate significantly in the range of 3-8.5. Even with a pH above 7, *S. cerevisiae* was able to degrade phenol and grew well; loss of degradation was low. Results demonstrated that the yeast efficacy values under the vast pH value of 3-8.5 were 93% and 92%, respectively. In a study by Park et al (20), phenol degradation by *Fusarium oxysporum* GJ4 was not affected by pH values under 6, and retardation of degradation was shown only in pH values above 7. Even at a pH above 7, *Fusarium*

Table 4. Optimization results of statistical analysis and confidence levels

pH	Phenol con.	Biomass con.	HRT	%Removal	Desirability
4.38	4615.20	1.50	11.86	0.8994	0.958
4.38	4615.15	1.50	11.98	0.8994	0.958
4.38	4619.10	1.50	11.79	0.8993	0.958
4.38	4614.36	1.50	11.63	0.8994	0.958
4.38	4638.81	1.50	12.23	0.9884	0.958
4.38	4617.55	1.50	12.32	0.8993	0.958

Table 5. Confirmation of experiment conditions and results

Run	Phenol concentration	Biomass concentration	HRT	pH	% Efficiency
A	4615.20	1.50	11.86	4.38	0.83
B	4615.20	1.50	11.86	4.38	0.81

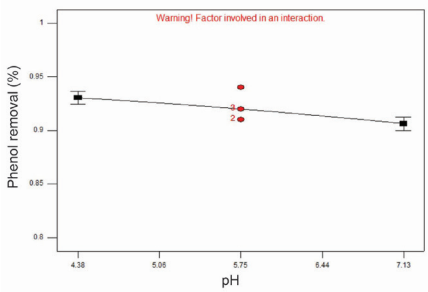

Figure 3. Effect of pH on phenol removal in ASBR reactor.

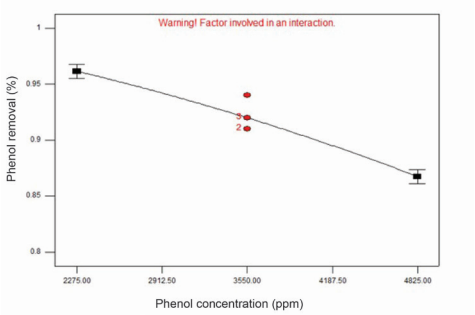

Figure 4. Effect of phenol concentration on phenol removal in ASBR reactor.

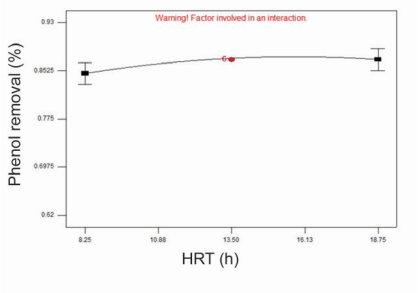

Figure 5. Effect of HRT on performance of batch system.

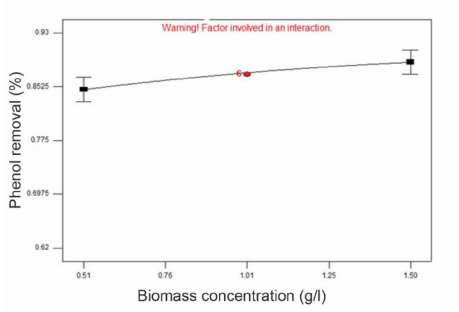

Figure 6. Effect of biomass concentration on phenol removal.

oxysporum GJ4 was still able to degrade phenol and grew well.

Effect of phenol concentration

At first, phenol degradation started once glucose concentration was significantly depleted. The degradation profile shows that the phenol degradation rate was initially high, but later it decreased until the end of the cycle, because

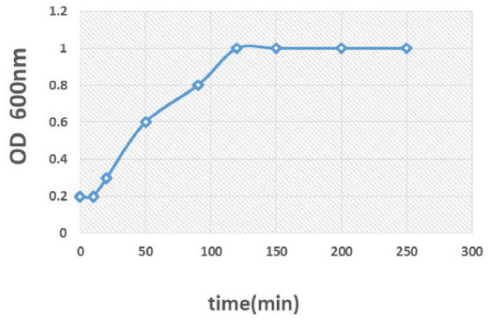

Figure 7. Optical density (OD) versus time growth curves for *S. cerevisiae* yeast

initially, the microorganism has a large amount of phenol available which is food for them, so they degrade it faster. Steady state conditions were reached after 3 days of continuous operation at 100 mg/L, with a phenol removal efficiency of 100%. Average phenol removal was close to 100% at an influent phenol concentration of 500 mg/L with each of the 3 support materials tested. The response of the degradation rate to the initial phenol concentration appeared to have 2 stages. At initial phenol concentrations between 500 to 1200 mg/L, increased phenol meant only a slight deterioration in the degradation rate, indicating insensitivity to phenol in that range. Beyond 2275 mg/mL (Figure 4), the negative effect of phenol was more marked. According to a study by Wang et al (21), the *Paecilomyces variotii* JH6 strain was able to utilize phenol as the sole source of carbon and energy at concentrations up to 1800 mg/l.

The effect of HRT

Biological wastewater treatment processes are cost-effective options, so that they not only can remove contaminants at a high efficiency with a low retention time, but also, the size and cost of the facility can be more appropriate. In an SBR system, the biggest fraction of cycle time is allocated to the aeration phase. HRT is defined as the full volume of the reactor divided by the total volume of wastewater fed into the reactor during one day. Under these operational conditions, the mean phenol removal efficiencies for a phenol concentration of 1000 mg/l were 66%, 75%, 86.5% and 99.9% in HRTs of 3, 8.25, 18.75 and 24 hours, respectively (Figure 5). Wei et al (22) reported that *Rhizobium* sp. CCNWTB701 could grow well in 900 mg/l of phenol and degrade 99.5% in 64 hours. It also degraded 78.3% in 66 hours in 1000 mg/l of phenol. In this study, the *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03 strains could grow well in 800 mg/l of phenol and degrade 99.5% and 97.6% within 45 hours, respectively.

Effect of biomass concentration

The initial sludge has an mixed liquid suspended solids (MLSS) concentration of 0.01 g/l. After the conditioning period, MLSS increased to 2 g/l. To test the influence of inoculums volume on the rate of phenol degradation, cell

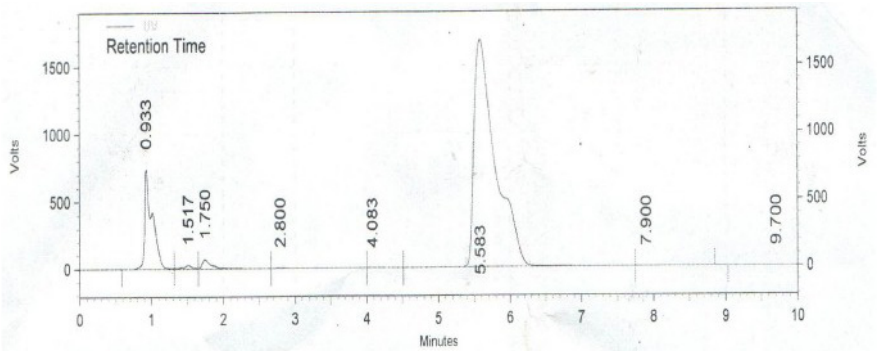

Figure 8. Chromatogram of HPLC analysis of phenol biodegradation by *S. cervisiea*

Table 6. HPLC analysis of catechol on Ascentis® RP-Amide application for HPLC

UV result name	Retention time	Area	% Area	Height	% Height
	0.933	5632782	13.5	741906	21.29
	1.517	247081	0.59	28392	1.12
	1.750	688103	1.65	72170	2.84
	2.800	85450	0.21	3068	0.12
	4.083	9030	0.02	484	0.02
	5.583	39116061	83.93	1692928	66.66
	7.900	18485	0.04	476	0.02
	9.700	7984	0.02	229	0.01
Total		41602976	100.00	2539653	100.00

suspensions with different densities (OD600 nm 0.2, 0.4, 0.5, and 0.7) were added to the phenol (1000 g/l)-containing glucose minimal medium under the same conditions. Results of this study showed a different rate of phenol degradation. An increase in the cell volumes resulted in an increased phenol degradation rate. Phenol degradation occurred after 3, 8.25, 13.5, 18.25, and 24 hours for cell densities with OD600 nm 0.2, 0.4, 0.5, 0.7 and 1, respectively (Figure 6).

When a higher amount of biomass was inoculated, tolerance to the phenol toxicity was observed at even higher initial concentrations of up to of 3550-4825 mg/L, although the growth was quite low. In this case, the degradation efficiency for phenol was also lower. Moreover, degradation was still observed for phenol in the concentration of 6100 mg/L; however, cell lyses could possibly occur and the biomass achieved was relatively low when the concentration exceeded 4825 mg/L. Therefore, it is possible to achieve efficient phenol biodegradation using S. *cerevisiae* with a low amount of sludge produced in the treatment of waste water containing phenol. In the same study by Duque et al (3), tolerance to phenol toxicity was observed at even higher initial concentrations (up to 2500 mg/L) when a higher amount of biomass was inoculated, although the growth was quite low. Moreover, degradation was still observed for phenol in the range of 3000-4000 mg/L, but it also lower.

Conclusion

Aqueous solutions of phenol are produced as a waste of industries and are discharged into the environment. The primary objective of this study was to investigate the performance of the ASBR when treating a synthetic wastewater containing high concentrations of phenol. It was revealed that pH had less of an effect on the phenol degradation rate by *S. cerevisiae* than bacteria did. Results concerning the effect of phenol concentrations revealed that these starving cells are able to promote phenol degradation efficiency slightly. Results also showed that the more HRT there is, the greater phenol removal efficiency will be. A direct relationship between phenol removal efficiency and biomass concentrations was also observed. Therefore, based on the results of the current study, this technology can be developed for the bio-decomposition of phenol.

Ethical issues

The authors certify that all data collected during the study is presented in this manuscript and no data from the study has been or will be published separately.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AZ wrote the manuscript, and performed the calculations

and experiments; MD and AS performed a portion of the experiment and helped in the calculations; AAN contributed to the experiment design and the preparation of final manuscript. HA, BM, AH and SJ participated in drafting the article and revising it.

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