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Impacts of Operating Parameters on Stability of Sequencing Batch Reactors Inoculated with Mixed Yeasts in Treating Edible Oil Refinery Wastewater

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Yeasts have distinct advantages in degrading many kinds of oil-containing wastewater produced from edible oil, olive oil or palm oil mill. Key operating parameters are crucial to set up a bioreactor with a stable and sound performance. In this study, mixed yeasts were inoculated in pilot-scale sequencing batch reactors (SBR) to treat edible oil refinery wastewater. Effects of carbon/nitrogen ratio, sludge retention time(SRT), as well as pH on the performance of SBR and yeast cell morphology were investigated. The results show that nitrogen is an important factor affecting yeast morphology and system stability. At a BOD:N:P ratio of 100:5:1, the SBR was operated successfully and steadily with SVI of 49.1±4.0 mL g^{-1}. However, at a BOD:N:P ratio of 100:2.5:1, yeast cell morphology transformed from yeast to long pseudohyphae which resulted in the pronounced increase of SVI and followed by extensive filamentous sludge; A mild increase of SVI and formation of short pseudohyphae were observed without perceptible change of pollutant removal efficiency when SRT was decreased from 60 d to 6 d; Increasing of operating pH from 5.5 to 7.0 led to a drastic increase of suspended solid and oil up to 612 and 2290 mg L^{-1}, respectively, in the effluent.

Key words: Oil degradation, Wastewater treatment, Yeast cell morphology, Nitrogen content, Sludge retention time, pH.

Due to the detrimental impact of oil contents on oxygen transfer efficiency in aerobic systems[1] and the inhibiting effects of long-chain fatty acids, such as oleic acid, the product of lipid hydrolysis on anaerobic systems[2,3], conventional aerobic or anaerobic biological treatments are not suitable for the direct treatment of wastewater containing high concentration of oily contents like edible oil refinery wastewater. To date, physico-chemical treatments, such as acidification, air flotation, coagulation, etc., followed by biological treatment have been mainly utilized for treating this kind of wastewater[4-7]. However, during the physico-chemical treatment, much sludge or scum in combination with oil is always produced and is difficult to deal with.

To overcome the above-mentioned problems of conventional technologies in treating oil-containing wastewater, yeasts instead of bacteria have been given more attention[8-11]. Chigusa *et al.*,[12] succeeded in employing nine yeast isolates to treat high-strength edible oil refinery wastewater. Zheng *et al.*[13] obtained similar results in treating this kind of wastewater with five different yeast strains. Yang *et al.*[14] isolated 257 yeast strains from municipal, inosine fermentation, papermaking, antibiotic fermentation, and printing and dyeing wastewater treatment systems and found that 16, 14, 55, and 11 strains produced lipase, protease, manganese-dependant peroxidase, and lignin peroxidase respectively. These findings

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demonstrated that direct biological treatment using yeast might be an effective solution for treatment of high-strength oil-containing wastewater or some kinds of refractory wastewater. In addition, the excess yeast biomass produced during wastewater treatment contains high concentrations of well-balanced amino acids, making it a potential single protein source (SCP) for fish breeding and poultry raising[15-17]. So yeasts exhibit the distinct advantages in degrading the oil-containing nontoxic wastewater. Thus far, yeast isolates have been tried to treat wider range of wastewater, such as the produced water from oil field containing high molecular weight-PAHs[18], olive mill wastewater[8], palm oil mill effluent[19], molasses wastewater[20], alcoholic distillery wastewaters[21], high strength fermentative wastewater[22] and wastewater with heavy metals[23]. However, previous studies always focused on the acquisition of yeast strains or the pollutant removal efficiency of the isolates, little attention has been paid to the necessary operating parameters of bioreactors and the stability of the treatment process. In fact, extensive filamentous sludge bulking was repeatedly encountered when yeast mixtures were applied to treating high-strength salad oil refinery wastewater, finally leading to operation failure in a continuously stirred tank reactor (CSTR)[24]. Thus, it is important to know how the key operating parameters of SBR affect the system efficiency and stability, and which can lead to the changes of yeast cell morphology and further the settleability.

In this study, pilot-scale SBRs were applied to evaluate the effects of three key operating parameters: carbon/nitrogen ratio, SRT as well as abrupt pH change on system performance and yeast cell settleability in the treatment of high-strength edible oil refinery wastewater. Also, changes of yeast cell morphology were traced microscopically to reveal the relationship between the yeast cell settleability and its morphology. The purpose of the present study was to establish a stable yeast treatment system with sound cell settle ablity and high pollutant removal efficiency.

MATERIALS AND METHODS

SBR system

Continuous wastewater treatment was carried out in pilot-scale SBRs (a cylinder of polymethylmethacrylate, effective volume 22.8 L) equipped with online pH and DO monitors. The schematic diagram was shown in Fig.1. The cyclic operation of SBR was controlled automatically by a program controller which manipulates the power supply of influent pump, air compressor and two magnetic valves. Valve 1, 2 is in charge of the effluent discharge and excess yeast biomass discharge, respectively. The pH of the mixed liquor was surveyed by the online pH monitor which sent a signal to the acid pump to adjust the real-time pH in accordance with the difference between the present pH and the preset pH in the refill phase. The air flow was controlled by a flowmeter to assure the DO of the mixed liquor is above 0.5mg/L .One treatment cycle included five operational phases: raw wastewater filling (1 h), aeration (9 h), settling (2.5 h), decanting (10 min) and idle phase (20 min). The drainage ratio is 1:2.

Analytical methods

COD and BOD_5 were determined respectively on a rapid analysis apparatus (CTL-12, Huatong Ltd., China; and Oxitop IS 12,WTW, Germany). pH was measured by a pH monitor (HDIC-3, TOA Electrononics. Ltd., Japan), and mixed liquor suspended solids (MLSS), SV_{30} (sludge volume in 30 min), oil, total nitrogen(TN), total phosphorus(TP) and suspended solids (SS)

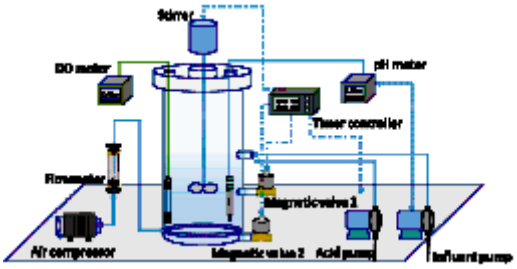

Fig. 1. The schematic diagram of Yeast-SBR system

were determined in accordance with the standard methods[25]. The yeast morphology during the SBR operation was observed on an scanning electronic microscope (FEI QUANTA 200, FIE, Holland, and light microscope (Axioskop 2 mot plus, ZEISS, Germany). The zeta potential was determined on a zeta potential meter (Zetasizer 2000, Malvern Instruments Ltd., UK). Triplicate samples were collected and assayed, and the data represent the mean values and standard deviations.

Characteristics of wastewater

Raw wastewater was collected from the adjusting tank in an edible oil refinery factory. The raw wastewater had the following characteristics: 1) high oil (4-16 g L^{-1}) and COD content (9-23 g L^{-1}); and 2) severe shortage of nitrogen (4.1-16.8 mg L^{-1}). Ammonium sulfate were supplemented into raw wastewater according to the experimental requirements. Supplementation of phosphorus was necessary because most of the phosphorus content in the raw wastewater were in the organic form and were not available for yeast cells. In order to exclude the effects of phosphate on the system, monopotassium phosphate was added into the raw wastewater to make the BOD: P ratio of 100:1.

Yeast strains and cultivation

Five yeast strains ((O2, *C. tropicalis* CGMCC 2.2158;O3, *C. boidinii Ramirez* CGMCC 2.2162; O3i, *R. rubra* (Demme) Lodde CGMCC 2.2156; O4, *C. utilis* CGMCC 2.2165; O5, *T. cutaneum* CGMCC 2.2164)), isolated from the soil at an edible oil refinery factory[24], and three commercial strains acquired from the China General Microbiological Culture Collection Center (G1, *C. lipolytica* CGMCC 2.1207; G2, *C. intermedia* CGMCC 2.1764; G3, *C. pseudolambica* CGMCC 2.1863), which were thought to potentially have abilities to degrade oil, were mixed together before cultivation.

Equivalents of yeast cells of different strains on slopes were taken and mixed in a flask with YPD media (yeast extract 5g, peptone 10g, dextrose 10g, pH 5.5, per liter deionized water). After 24h cultivation (28°C, 175r/m), the mixed yeast culture was transferred into an open tank containing edible oil refinery wastewater with sufficient supplement of ammonium sulfate and monopotassium phosphate and the culture conditions are similar to the procedure described by Zheng *et al.*,[24] with some modifications. It should be noted here that sodium propionate used to inhibit the growth of mold and/or bacteria was not added[13,24,26] and the pH of the mixed liquor was not further controlled when it was adjusted to 5.5 during the filling process in this study. In one cycle, 9 h aeration was followed by 2.5 h settling, and 0.5h supernatant discharge and raw wastewater refill.

Operation of the SBR system

Effects of carbon/nitrogen ratio

SBRs were operated under the BOD:N:P ratio of 100:5:1 and 100:2.5:1 for 20 days independently. The pH of the mixed liquor was not controlled after it was adjusted to 5.5 using an online pH monitor during the filling phase. The mean concentrations of COD and oil in feed wastewater were kept at 15 g L^{-1} and 10 g L^{-1}, respectively, to keep the BOD load constant (about 0.5 kg BOD $kg^{-1}MLSSd^{-1}$) and to evaluate the system's ability to treat high-strength edible oil refinery wastewater. The MLSS, SVI, COD and oil content were examined every day.

After 20 days of running under BOD:N:P ratio of 100:2.5:1, the ratio was adjusted to 100:5:1 and the second SBR was operated for another 20 days to evaluate the recovery ability of nitrogen supplement to system performance.

Effects of SRT

Under a BOD:N:P ratio of 100:5:1, the system was operated for 18 days at an SRT of approximately 60 d by discharging 540 mL of mixed liquor in idle phase every three days from the SBR. Afterwards, the SRT was shortened to 6 d for 12 days by discharging 800 mL of mixed liquor every running cycle. During the whole operation, the BOD load was kept at approximately 0.5 kg BOD $kg^{-1}MLSSd^{-1}$.

Effects of the pH

When the SBR reached the stable operation state at the BOD:N:P ratio of 100:5:1, the pH of the mixed liquor in the filling phase was increased to 6.0 for 2 days, and then up to 7.0 from the initial 5.5 to investigate the effects of abrupt increasing pH on system stability. Three days later, the pH was adjusted back to 5.5. pH, SS and Oil content of both influent and effluent were determined every two days during the operation. In order to investigate the mechanism of the fluctuation of SS and oil content in effluent, the supernatant in SBR was taken to determine the zeta potential before it was discharged out.

RESULTS

Preparation of yeast biomass in an open tank

Yeast cultures with an MLSS of 18.6±2.3 g L^{-1} and SVI of 38.7±10 mL g^{-1} were harvested after 7 d cultivation. At the end of cultivation, most of yeast cells existed as yeast form, clumping together to form flocs (Fig. 2A).

Effects of carbon/nitrogen ratio

SBRs were operated under two carbon/nitrogen levels: high condition, BOD:N:P=100: 5:1 and low condition, BOD:N:P=100:2.5:1, independently. As shown in Fig. 3, the system was in a healthy state (SVI, 49.1±4.0 mL g^{-1}; MLSS, 17.4±1.5 g L^{-1}) under the high nitrogen condition. Microscopic observations indicated that the dominating microorganisms were also unicellular yeasts.

When the SBR was running under BOD:N:P ratio of 100:2.5:1, however, the SVI increased gradually from 50.1 to 160.6 mL g^{-1} in the following two weeks, followed by a sharp increase in the next several days, leading to significant washout of yeast biomass. After 18 d operation, the SVI climbed up to 484.8 mL g^{-1}, and the MLSS in the SBR decreased from 15.0 to 2.0 g L^{-1}, leading to the final collapse of the system. Under this low nitrogen condition, the yeast morphology changed from yeast to filamentous form on day 9 (Fig. 2B), and finally, most of yeast cells became long pseudohypha on day 18 (Fig. 2C). After 18 days, the BOD:N:P ratio was adjusted back to 100:5:1. However, the settleability of yeast biomass was not improved in the following operation, suggesting that it is difficult to reverse the cell morphology switching simply by supplementing

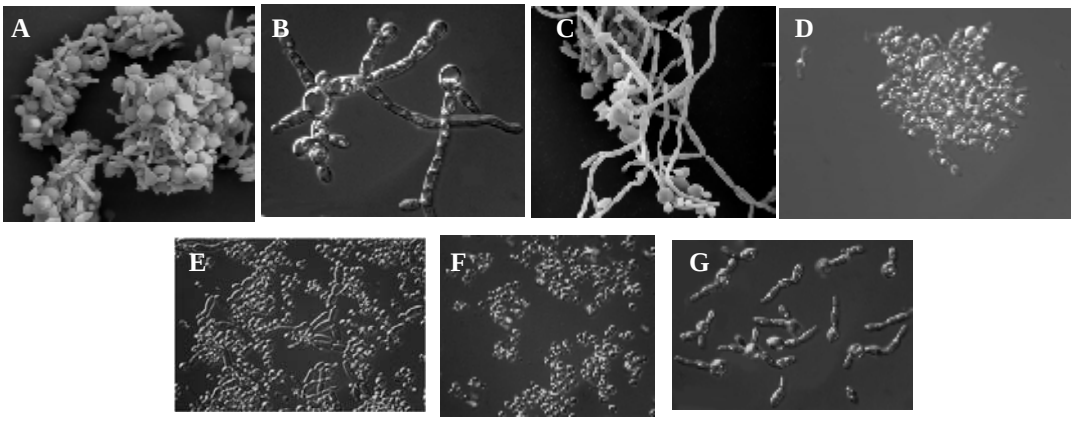

Fig. 2. Images of yeast cell morphologies. A .SEM image of yeast flocs formed during cultivation in YPD media; B. Microscopic image of yeast cells on day 9 under low carbon/nitrogen condition; C. SEM image of yeast cells on day 18 under low carbon/nitrogen condition;D and E. Microscopic image of yeast cells at SRTs of 60 d and 6 d; F. Microscopic image of yeast cells in the sludge after 2 hours of sedimentation at operating pH of 7; G. Microscopic image of yeast cells in the decanted water after 2 hours of sedimentation at operating pH of 7.

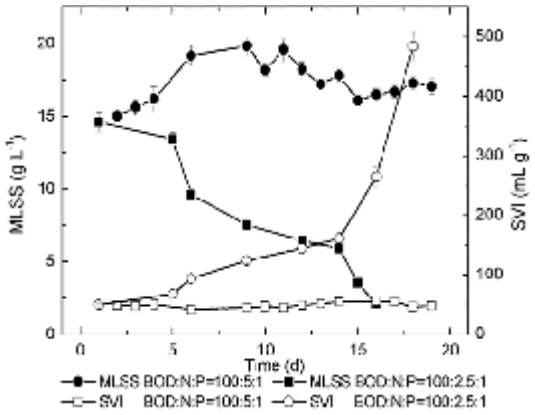

Fig. 3. Comparison of MLSS and SVI under different carbon/nitrogen conditions

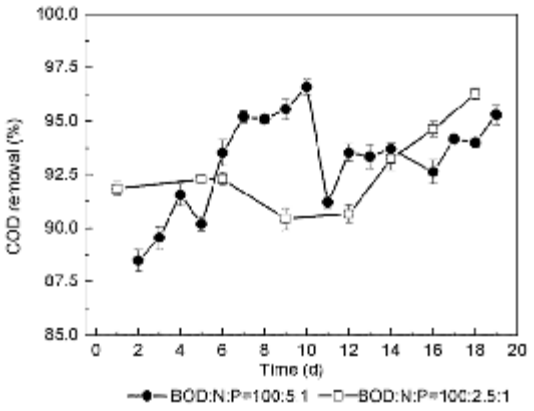

Fig. 4. Comparison of COD removal under different carbon/nitrogen conditions.

sufficient nutrition after undergoing the state of nitrogen scarcity.

Fig. 4 compares the COD removal under the two carbon/nitrogen conditions. The SBR systems running under high and low nitrogen conditions showed high removal of COD(93.2±2.3% and 92.8±2.0%, respectively) and no significant differential was found. And furthermore, by comparing the results in Fig. 3 and Fig. 4, under the low nitrogen condition, the COD removal was enhanced with the increase of SVI in spite of the significant loss of yeast biomass. The effluent COD was decreased to approximately 500 mg L^{-1} as compared with that of 700 mg L^{-1} under the high nitrogen condition.

Effects of SRT

The SRT determines the average age of yeast cells in a reactor. The effect of SRT on the settleability of yeast biomass was investigated, and the result is shown in Fig. 5. When the SRT was decreased from 60 to 6 d, the SVI increased from 46.1±4.9 to 78.1±5.1 mL g^{-1}. As shown in Fig. 2D and Fig.2E, the yeast morphology was a little different under the two SRTs. Under the SRT of 60 d, the dominant morphology was round/oval unicells, which attached with each other to constitute condensed flocs. This was similar to that observed during yeast cultivation (Fig. 2A). By comparison, during a shorter SRT of 6 d, bacilliform yeast consisting of several unseparated yeast cells became dominant (Fig. 2E), which possibly caused the increase of SVI. Such an increase of SVI, however, did not affect the settleability of yeast biomass. The SBR was operated successfully for 30 days with SVI of 60.9±17.0 mL g^{-1} and COD and oil removals of 92.1±2.4% and 99.7±0.1%, respectively with an average influent COD of 15 g L^{-1} and oil concentration of 10 g L^{-1}.

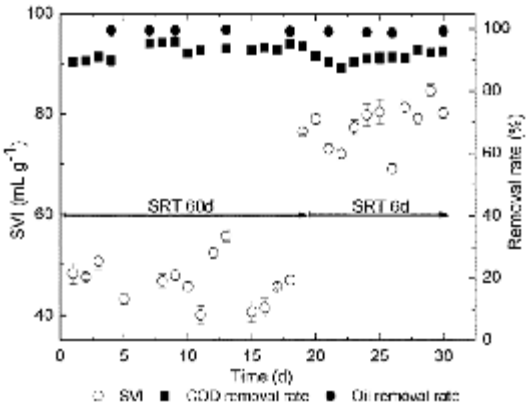

Fig. 5. Effects of SRT on COD and oil removal and SVI at BOD:N:P ratio of 100:5:1.

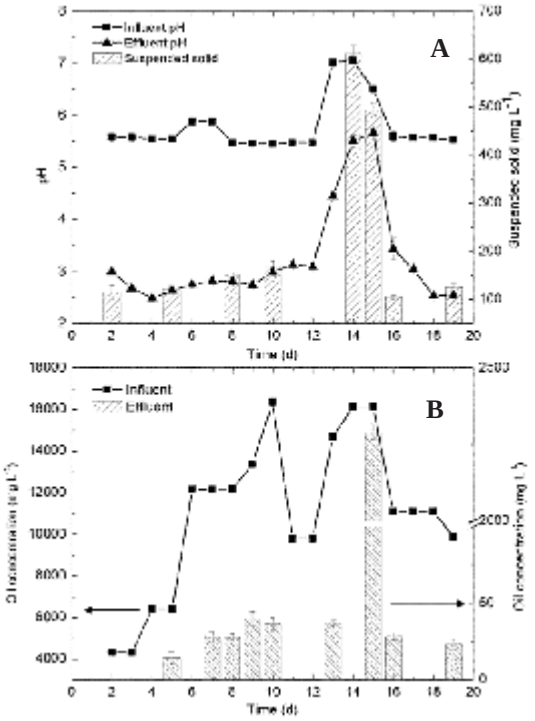

Fig. 6 Effects of pH on the stability of SBR. (A) Effluent pH and SS; (B) Oil removal efficiency.

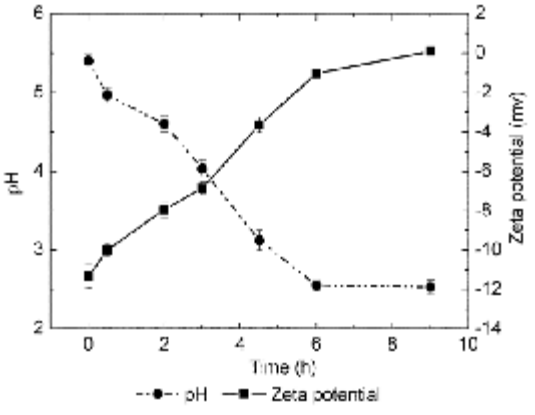

Fig. 7 Changes of zeta potential and pH of mixed liquor in SBR during one operation cycle.

Effects of pH

During the treatment of wastewater, when the operating pH (the pH at the filling phase) was adjusted to 5.5 using sulfuric acid, the effluent pH decreased to 2.5-3.0 at the end of the cycle. If the system could be operated under a higher pH(the pH of raw wastewater is 8.0), the cost for pH adjustment could be reduced. Therefore, the possibility of operating the system at a higher pH was investigated (results are shown in Fig. 6). Increasing the operating pH from 5.5 to 6.0 resulted in an increase of effluent SS from 120 to 170 mg L^{-1} and oil content from 16 to 36 mg L^{-1}. However, when the pH was increased to 7.0, the SS and oil content increased drastically to 612 and 2290mg L^{-1}, respectively, within the subsequent 2-3 operation cycles. As shown in Fig. 2F, unicellular round/rod yeast cells were dominated in the settled yeast biomass, indicating that morphology switching did not occur. High concentrations of round yeast were observed in the decanted water after 2 hours of sedimentation, suggesting that the round yeast might be easily washed out from the system under higher pH conditions (Fig. 2G).

DISCUSSION

Effects of Nitrogen deficiency in wastewater on stability of SBR system

Sudbery *et al.*[27] reported *Candida albicans* can grow in at least three different morphologies: yeast, pseudohyphae and hyphae, and a variety of environmental conditions may induce the morphology switching from yeast to pseudohyphae or hyphae. For example, limitation of nitrogen could induce the occurrence of pseudohyphae when this strain grew on a solid medium; while the presence of N-acetylglucosamine or pH 7.0 and 37°C led to the transformation of cell from yeast to hyphae. However, few literatures have been reported on the yeast strains mentioned in present study and the morphology of yeast cells survived in wastewater remained unclear. The SBR system is a complex of a variety of environmental and nutritional conditions. the good settleability of yeast biomass depends on the yeast cell morphology. Thus, it is worthwhile to further investigate the factors which induce the morphology transition of yeast cells.

In our previous studies, the experiments were carried out in laboratory-scale SBRs. Significant increases of SVI were observed when edible oil refinery wastewater was treated for several days without supplementation of ammonium sulfate, followed by extensive filamentous sludge bulking[28]. According to the experimental guidelines for distinguishing hypha and pseudohypha proposed by Sudbery et *al.*,[27], the filamentous forms can be concluded as hypha because there are constrictions at septal conjunctions and the sides of the elongated compartment are parallel. On the contrary, pseudohypha rather than hypha were observed in present study. Although the same yeast strains were inoculated in these SBRs, the distinct yeast cell morphology was observed. The possible reason is strain selection happening in the running of SBRs which results in the different predominant yeast strains. Under the lower nitrogen condition, certain yeast strain grows in hyphal form, whereas other strain grows in pseudohyphal one. In fact, we have proved that seven out of ten strains were washed out from the SBRs during the long time operation[29]. Since the final consequence of these two yeast forms was severely bulking and increased SVI, the suitable nitrogen concentration is an important factor affecting the stable operation of SBR. Further research should be carried out to elucidate the cell morphology of dominant yeast species under different nitrogen conditions.

Intriguingly, despite the poor settleability of yeast biomass and the low MLSS under the lower nitrogen condition, the system could also maintain a significantly high COD and oil removal. The effluent COD is even lower than that under high nitrogen condition in the following short period. It is possible that the filamentous morphology of yeast made it easier to assimilate organic substrates with small molecular weight. Recent researches have demonstrated that the limited flamentous bulking induced by low dissolved oxygen(DO) in activated sludge system could improve the quality of effluent of wastewater treatment[30, 31].

Effect of SRT on stability of SBR system

SRT indicating the mean residence time of microorganisms in the reactor is a key parameter for the design of conventional activated sludge system or membrane bioreactor. High SRT means

the survival of slowly growing microorganisms and consequently, the higher diversity of microbial communities. In recent years, effects of SRT on the pollutants removal performance and sludge characterization of different reactors were investigated extensively by many researchers[32-38]. High SRT was not only propitious to the growth of nitrifiers, but also benefit to increased oxygen transfer efficiency, improved biomass particle size distribution and enhanced removal of many emerging contaminants such as pharmaceuticals, endocrine disrupting compounds[36,39]. Ng and Hermanowicz[40] reported concentrations of protein and carbohydrates in the extracellular polymeric substances (EPS) decreased with decreasing SRT in the completely mixed activated sludge system and a reduction of EPS at shorter SRT deteriorated sludge settling properties because sludge flocs were smaller and weaker. Meanwhile, higher SRT can reduce the amount of sludge produced so as to save the cost for the sludge handling and disposal. The findings of Masse *et al.*[41] showed sludge production in a submerged membrane bioreactor (SMBR) decreased from 0.31 to 0.13 gVSS g^{-1}COD as SRT increased from 9 to 110 days. The above-mentioned results about SRT all obtained from the activated sludge, the effects of SRT on the yeasts inoculated in SBR remain to be elucidated.

Our present results showed the size of yeast flocs under SRT of 60 d were larger and denser than those under SRT of 6d, indicating that there is a similar rule between activated sludge and yeast. We speculated that under the higher SRT, more EPS was bounded onto the yeast cells and made them form the flocs, which have the better settleability than separated yeast cells under the lower SRT. On the other hand, some round yeast cells were observed microscopically in the effluent of the SBR system when the SRT was decreased from 60 d to 6d indicating some yeast strains was washed out from the SBR.

Although slight changes in term of yeast cell morphology, floc distribution and SVI were observed when the SRT was altered from 60d to 6d, the COD and oil removal rate were stable and the increasing SVI didn't deteriorate the stability of system. According to the findings, we can run the system under a broader range of SRT which depends on the specific purpose. If the aim is to harvest yeast cells, we can select a lower SRT. Contrarily, if the aim is just to treat the wastewater, a higher SRT can be chosen as to minimize the excess yeast biomass.

Effect of pH on stability of SBR system

It is well known that cell charges, which are mainly controlled by solution pH, affect interactions between cells. Changes of zeta potentials and pH of the mixed liquor in the SBR were followed in one operation cycle, and the results are shown in Fig. 7. At the beginning, the pH was adjusted to 5.5, and the corresponding zeta potential was -12 mv. With the progression of treatment, the pH decreased gradually, and the final pH was 2.5. Correspondingly, the zeta potential increased gradually, and the final value was near zero, at which repulsion among yeast cells became the lowest. When the operating pH was increased to 7.0, however, the final pH of each cycle was approximately 5.5, and this could correspond to a zeta potential below -10 mv. Under this scenario, the negatively charged yeast cells would repel each other, deterring the formation of flocs and resulting in the poor settleability of yeast cells. Therefore, the deterioration of settleability at the operating pH 7.0 was caused by a different mechanism from that under low nitrogen conditions (by hypha). However, the pollutants in the edible oil refinery wastewater are mainly comprised of long chain fatty acids, which can be more easily deprotonized at higher pH. The negatively charged yeast cells would not be able to easily capture the negative fatty acids, which might be the main reason for the deterioration of oil removal shown in Fig. 6b.

In our previous study, mixed yeasts were inoculated into six laboratory-scale SBRs under different pH (4, 5, 6, 7, 8 and 9 in filling phase) individually. The results showed under the pH 6 high COD removal and MLSS were achieved and the yeast was the dominant microbes in the system. However, when the running pH increased to 7, although high MLSS was maintained, the COD removal rate decreased drastically. If the running pH is higher than 7 for a long time, bacteria and protozoa became the predominant species and the system ran into collapse. Obviously, the abrupt increasing pH in this study put a different effect on the SBR system. Meanwhile, the negative effects can be removed by changing pH back to 5.5.

CONCLUSION

Eight yeast strains were inoculated into pilot-scale sequencing batch reactors (SBRs) to directly treat the edible oil refinery wastewater successfully and high COD and oil removal were achieved in 30 days operation. Our results revealed for the first time the effects of carbon/nitrogen ratio, SRT and abrupt pH on the performance and stability of the SBRs, especially on the relationship between these parameters and yeast cell morphology. It has been proved that nitrogen condition is an important parameter in good working operation of SBRs. Shortage of nitrogen in raw wastewater resulted in the certain yeast cells growing in the long pseudohypha form and further the irreversible bulking of system. Broad rang of SRT (6-60 d) can be adopted to treat the wastewater without changes of COD and oil removal efficiency, but slight changes of cell morphology and SVI were observed which didn't affect the stability of SBRs. Abrupt pH increase to 7 led to the performance deterioration of the SBRs in a short time and the negative effects can be eliminated by adjusting the pH back to 5.5. Our findings will be expected to establish a stable and sound SBR system in which yeasts are the predominant microbes to treat oil-containing wastewater.

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