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USING AMMONIUM-TOLERANT YEAST ISOLATES: *CANDIDA HALOPHILA* AND *RHODOTORULA GLUTINIS* TO TREAT HIGH STRENGTH FERMENTATIVE WASTEWATER

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ABSTRACT

Two ammonium-tolerant yeast strains were isolated from sludge samples contaminated with monosodium glutamate manufacturing wastewater and were identified as *Candida haplophila* and *Rhodotorula glutinis*. The tolerance of the two yeast isolates to ammonia and their chemical oxygen demand (COD) removal performances were evaluated under batch and bench-scale conditions. The mixture of the two isolates was found to grow well in an artificial medium containing 25% (NH₄)₂SO₄ and could effectively remove COD from monosodium glutamate wastewater even when the concentrations of NH₄⁺-N and free NH₃-N reached as high as 18977 and 879 mg l⁻¹ respectively. A fixed-bed yeast reactor, which was initially inoculated with the yeast mixture, permitted a constant COD removal rate of over 80% during a period of near 2-month continuous running even when the influent COD was increased from 8000 to 25000 mg l⁻¹. The effluent was accompanied with suspended solids (SS) of over 4500 mg l⁻¹, which was mainly composed of yeast cells and could be considered as a source of animal forage additive. The residual COD of effluents from the yeast reactor could be further reduced to under 500 mg l⁻¹ by a combination process of activated sludge treatment and coagulation technologies.

Keywords

Yeast, monosodium glutamate (MSG), wastewater treatment, ammonium

INTRODUCTION

In China, there are more than 600 monosodium glutamate (MSG) manufacturing plants producing nearly 1 million ton MSG annually. MSG wastewater is characterized by high concentrations of chemical oxygen demand (COD) (10000-30000 mg l⁻¹), ammonium (15000-25000 mg l⁻¹ NH₄⁺-N), and sulfate (15000-30000 mg l⁻¹), which makes it very difficult to be treated with conventional biological treatment processes in spite of the high biochemical oxygen demand (BOD) to COD ratio (>0.8). Anaerobic techniques are believed to be one of the most efficient processes for treating high strength organic wastewater. However, the toxic inhibition of ammonia took place to most anaerobes in spite of pH control when the concentration of NH₄⁺-N was higher than 3000 mg l⁻¹ [1]. The conventional aerobic process was also significantly affected by high concentration of NH₄⁺-N. Li et al [3] and Zhao [3] reported that the COD removal in an activated sludge process declined from 95.1 to 78.1% when the NH₄⁺-N concentration was increased from 50 to 800 mg l⁻¹. The common practice in China is to dilute wastewater to eliminate the microbial inhibitions caused by high concentrations of

ammonia or sulfate [4-6], which resulted in high operation costs and waste of precious water resources. Therefore, a technique employing microorganisms that can tolerate high strengths of NH₄⁺-N and sulfate is required to solve the problem.

From the 1970s, there have been many reports on the application of yeast to wastewater treatment under aerobic and open conditions [7-10]. Compared with the conventional biological wastewater treatment process in which bacteria are predominant, yeast treatment technology which permits a high COD load and endures high osmotic pressure makes it a promising alternative for treating MSG wastewater. In this study, yeast isolates capable of growing directly in MSG wastewater were obtained through screening and their tolerance to ammonia and COD removal performances were evaluated under batch and bench-scale conditions.

MATERIALS AND METHODS

Wastewater Characterization

The characteristics of MSG wastewater samples

collected from an MSG manufacturing factory in Tianjin, China are shown in Table 1. It is obvious that the sample contained high concentrations of COD, $\text{NH}_4^+\text{-N}$, SO_4^{2-} , suspended solids (SS), and the pH was low.

Table 1. Characteristics of MSG wastewater.

Determination items	Concentration (mg l^{-1})
COD	24125
Total organic carbon	7411
BOD_5	18000
SS	1200
Total phosphate	37.3
SO_4^{2-}	20706
$\text{NH}_4^+\text{-N}$	18977
Protein	960
Reducing sugar	5981
Total sulphur	12%
pH	2.0
Temperature	35°C

Analytical Methods

COD, TOC, SS, $\text{NH}_4^+\text{-N}$, SO_4^{2-} and total phosphate (TP) were determined according to Standard Methods [11]. The concentration of soluble protein was detected by Bradfords method [12] and the reducing sugar was determined according to 3, 5-Dinitrosalicylic acid method (DNS method) [13]. The ratio of NH_3 to NH_4^+ is pH and temperature dependent of the solution. The un-ionized ammonia fractions (NH_3) were calculated by the concentrations of $\text{NH}_4^+\text{-N}$ in the solution [14].

Media for Yeast Isolation and Preservation

Selective medium 1: glucose 20 g, yeast extract powder 5 g, peptone 10 g, sodium propionate 0.25%, agar powder 2%, tap water 1000 ml, pH 4.5, adding streptomycin to 30 mg l^{-1} just before use. This medium was used for yeast screening.

Selective medium 2: MSG wastewater sample 1000 ml, sodium propionate 0.25%, agar powder 2%, pH 4.5, adding streptomycin to 30 mg l^{-1} just before use. This medium was used for yeast screening.

YPD medium: glucose 20 g, yeast extract powder 5 g, peptone 10 g, agar powder 2%, tap water 1000 ml, pH 4.5. This medium was used for yeast cultivation and preservation.

Nitrogen free minimal medium: glucose 20 g, K_2HPO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, yeast extract powder 0.1 g, distilled water 1000 ml, pH 7.0. This medium was used for evaluating the endurance of yeast to $(\text{NH}_4)_2\text{SO}_4$.

Enrichment, Isolation, and Identification

Yeast enrichment and isolation were followed by the

methods proposed by Chigusa et al. [10]. Sludge samples were collected from several spots contaminated with MSG wastewater. One gram of sludge sample was inoculated into 150 ml MSG wastewater whose pH was adjusted to 4.5-5.0, and cultivated at 28°C, 170 rpm on a reciprocal shaker. Half volume of the culture was removed and supplemented with new MSG wastewater once a day. After enrichment cultivation for 10 days, 10 ml of the culture was transferred to 150 ml new MSG wastewater containing 30 mg l^{-1} streptomycin and cultivated for 2 days. Then 0.2 ml of the culture was transferred to agar plates containing selective medium 1 and selective medium 2 respectively and cultivated for another 2 days. Yeast colonies with different shapes were selected from the agar plates and repeatedly transferred onto new plates until the cultures were pure. Stock cultures were stored on YPD plates at 4°C and periodically subcultured.

Yeast strains isolated by the above procedure were identified according to their respective morphological and physiological characteristics under different carbon or nitrogen sources [15].

Endurance of Isolates to Ammonia

The endurance of mixed isolates to different strengths of ammonia-nitrogen was tested in both sterile artificial medium and nonsterile actual wastewater. The nitrogen free minimal medium was added with $(\text{NH}_4)_2\text{SO}_4$ to obtain an $(\text{NH}_4)_2\text{SO}_4$ concentration varying from 0.5 to 50% and sterilized by bacteriological filters (pore size, 0.4 μm). MSG wastewater containing 18977 mg l^{-1} of $\text{NH}_4^+\text{-N}$ was diluted by the ratios of 1:9, 1:2 and 1:1 to achieve different ammonia-nitrogen strengths. One milliliter of mixed yeast culture from liquid YPD medium was used as inoculum. Flasks were prepared in triplicate for each gradient and incubated at 28°C, 170 rpm in a shaker. Samples were taken periodically. The growth of yeast in artificial medium was evaluated by measuring the absorbance at 660 nm on a spectrophotometer, and the growth in MSG wastewater was determined by assaying mixed liquor suspended solids (MLSS).

Effect of Initial pH on COD Removal of MSG Wastewater by Isolates

Mixed yeast culture of 10 ml from YPD medium was inoculated into 100 ml diluted MSG wastewater (1:9) of which the pH was adjusted to 2.0-9.0. Flasks were prepared in triplicate for each pH gradient and cultivated in a reciprocal shaker at 28°C, 170 rpm for 48 h under nonsterile conditions. Samples were taken periodically to assay the COD variation.

Batch Treatment of MSG Wastewater by Isolates

One liter wastewater with various dilution ratios (1:9, 1:2, 1:1 and raw wastewater) was respectively inoculated with wet yeast cells at the inoculum of MLSS to COD, 1:1.5 and cultivated at 28°C, pH 4.5 in a 2 l-tank under nonsterile

conditions. Air was supplied with an air pump controlled by an air flow meter, and samples were taken periodically for COD measurement.

Bench Scale Treatment System

Ethylene fibers (5 cm in length) fixed on plastic rings (ϕ 5 cm, 10 cm between the neighboring rings) were hung in a lucite cylindrical column (effective volume of 12.2 l) as biocarriers (shown as Figure 1). The column was continuously fed with diluted wastewater (1:9) from the bottom after 1 l mixed yeast culture was inoculated to the column reactor. The reactor was thermo-regulated at 28°C and operated at a hydraulic retention time (HRT) of 40 h. Air was supplied from the bottom of the reactor with an air pump controlled by a flow meter. In all the steady state runs, the dissolved oxygen concentration (DO) in the reactor was controlled to a range of 2-4 mg l⁻¹ to ensure an aerobic treatment. The strength of wastewater was gradually increased through decreasing the dilution ratio of wastewater after the COD removal of effluent was stable. Table 2 shows operation conditions.

RESULTS AND DISCUSSION

Characteristics and Identification of Isolates

Two strains of yeast (WG1 and WG2) were isolated and purified. The morphological and physiological characteristics of every single isolate were studied and their abilities of

utilizing different organics as sole carbon or nitrogen source were detected (shown in Table 3). According to the method proposed by Barnett *et al.* [15], WG1 and WG2 were identified as *Candida haplophila* and *Rhodotorula glutinis*, respectively. Considering mixed strains are more suitable for practical application, the two yeasts were cultured together as seed yeast for the following tests.

Endurance of Mixed Yeast to Ammonia

To evaluate the tolerance of the two isolates to ammonia-nitrogen, the kinetic behaviors of *Candida haplophila* and *Rhodotorula glutinis* growing in different concentrations of (NH₄)₂SO₄ were investigated in both sterile artificial medium and nonsterile wastewater. The results are shown in Figure 2 (a) and (b).

From Figure 2 (a), it was found that the mixed yeast strains grew at the maximum rate before 24 h when the concentration of (NH₄)₂SO₄ was in a range of 0.5-5% (*i.e.*, 1029.6-10296 mg l⁻¹ NH₄⁺-N). The growth rate showed a small decrease when the dose of (NH₄)₂SO₄ was increased to 10% (20592 mg l⁻¹ NH₄⁺-N) and a significant repression of yeast growth was observed under 25% (NH₄)₂SO₄ (51476 mg l⁻¹ NH₄⁺-N). The two yeast strains couldn't grow under 30% (NH₄)₂SO₄ over a period of 48 h. Therefore, a concentration of 25% (NH₄)₂SO₄ might be the upper limit for the growth of the yeast mixture. Few reports were found about the effects of NH₄⁺-N on the growth of aerobic bacteria. Inhibition of anaerobic bacteria under high ammonia concentrations,

Figure 1. Yeast treatment system for MSG wastewater (1. Feed tank, 2. Volumetric pump, 3. Biofilm column reactor, 4. Air flow meter, 5. Air pump, 6. Thermoregulator, 7. Effluents).

Table 2. Operation conditions of bench-scale test.

Feeding rate (l d ⁻¹)	Influent COD (mg l ⁻¹)	Influent pH	Air flow (l min ⁻¹)	HRT (h)	Temperature (°C)
7.2	8000~25000	2.0~3.0	30~100	40	28

Table 3. Characteristics and identification of isolates.

Items	WG1	WG2	Items	WG1	WG2
<u>Morphological characteristics</u>			Sucrose	-	+
Vegetative cell	Budding	Budding	Maltose	-	+
Size (μm)	2-4	2-10	Cellobiose	+	+
Filaments	-	-	Trehalose	+	+
<u>Characteristics in liquid medium</u>			Lactose	-	-
Membrane-forming	+	+	Melibiose	-	-
Spore-forming	-	-	Raffinose	-	+
			Melizitose	-	+
<u>Physiological characteristics</u>			Soluble starch	-	-
Sugar fermentation			D-xylose	-	+
D-Glucose	+	-	L-Arabinose	-	-
D-Galactose	+	-	D-Arabinose	-	
Maltose	-	-	D-Ribose	-	-
Sucrose	+	-	L-Rhamnose	-	-
Lactose	-	-	Glycerol	+	+
Raffinose	-	-	Erythritol	-	-
			Ribitol	-	-
Arbutin splitting	+		Galactitol	-	-
Vitamin-free medium	-	+	D-Mannitol	+	-
Urea hydrolysis	-	+	D-Glucitol	-	
Starch-like compounds	-	-	Salicin	+	
Growth at 37°C	-	-	DL-Lactate	-	
			Succinate	+	+
Assimilation			Citrate	+	+
Galactose	+	+	Inositol	-	-
Sorbin	-		Nitrate	+	+
			assimilation		
Results of identification		WG1: <i>Candida halophila</i>		WG2: <i>Rhodotorula glutinis</i>	
+, positive reaction; -, negative reaction

Figure 2a. Growth of mixed yeast in artificial medium containing different concentrations of $(\text{NH}_4)_2\text{SO}_4$ (○, 0.50%; ▲, 5%; ◆, 10%; ■, 15%; ●, 20%; □ 25%).

Figure 2b. Growth of yeast under different strength of MSG wastewater (wastewater to water) (◆, 1:9 dilution; ■, 1:2 dilution; ▲, 1:1 dilution; ●, no dilution).

however, has often been reported. Anthonisen *et al.* [1] found that methane-forming bacteria were inhibited in spite of pH control when the total ammonia was over 3000 mg l⁻¹. Therefore, the mixture of *Candida haplophila* and *Rhodotorula glutinis* could endure the total ammonia 3-17 times as high as the methane-forming bacteria.

Figure 2 (b) shows that the MLSS increased with time under each dilution ratio, and a higher MLSS corresponded to a lower dilution ratio. From Figure 2 (b), the kinetic trends of yeast growth under different dilution ratios were obtained. There was no noticeable lag phase of growth. Yeast went into fast growth phase immediately after inoculation, and then stopped into a stable phase (except for the case of no dilution). During the rapid growth phase, the growth rates of yeast were basically in proportion to the concentrations of COD in

the solution. The higher the initial COD, the higher the growth rate. No significant inhibition of growth was observed even under the condition of no dilution (18977 mg l⁻¹ NH₄⁺-N, 20706 mg l⁻¹ SO₄²⁻), indicating that *Candida haplophila* and *Rhodotorula glutinis* were suitable for treating MSG wastewater directly.

Effect of pH on COD Removal

The percent COD removals following 48 h treatment under different pH conditions were obtained and shown in Figure 3. According to Anthonisen *et al.* [1], the growth of microorganisms could be inhibited both by total ammonia and by free NH₃, and the latter was far more toxic than NH₄⁺.

Figure 3. Effect of initial pH and NH₃ concentration on COD removal (■, COD removal (%); ◆, NH₃ (mg l⁻¹)).

It has been a well-known fact that the concentration of free NH_3 in a solution was dependent on pH, temperature and NH_4^+ concentration. To evaluate the effect of pH and NH_3 on COD removal, the concentration of free NH_3 under each pH condition was also calculated and shown in Figure 3. The activities of yeast were almost completely inhibited at pH 2, suggesting that a strong acidic condition was harmful to the yeast mixture. The pH effect was not noticeable in a pH range of 4-9, although the concentration of NH_3 in the solution varied from 1.6 to 879.1 mg l^{-1} . The percent COD removal was stable at more than 80%. According to Anthonisen *et al.* [1], 10-150 mg l^{-1} of $\text{NH}_3\text{-N}$ was inhibitory to both nitrifying and heterotrophic bacteria. The result of Figure 3 further demonstrates that the tolerance of *Candida haplophila* and *Rhodotorula glutinis* to free NH_3 was very strong. Since the pH of MSG wastewater was as low as 2, the initial pH in the following tests was adjusted to ca. 4 to ensure a final pH of 5 to 7.

COD Removal Under Different Dilution Ratios

COD removals under different wastewater dilution ratios were investigated in a series of batch tests. The initial pH of every reactor was adjusted to 4.5 before the yeast mixture was inoculated. The inoculums were adjusted in proportion to COD concentrations to get similar reaction

rates. Variations in COD concentrations as well as COD removal percent against time are plotted in Figure 4. It was found that the final COD removal was the same 84.5% when wastewater was diluted from 1:1 to 1:9 in spite of the large variations of the concentrations of COD and $\text{NH}_4^+\text{-N}$. However, the percent COD removal for the solution without dilution was still increasing in the following 48-h treatment. From Figure 4, the COD decreasing rates ($\text{mg l}^{-1} \text{h}^{-1}$) at any time could be acquired by regressing calculation. The initial COD decreasing rate was 240, 552, 631 and 482 $\text{mg l}^{-1} \text{h}^{-1}$ corresponding to wastewater with 1:9, 1:2, 1:1 dilution and no dilution. Except for the case without dilution, the initial COD decreasing rate was proportional to the initial COD concentration in the solution, indicating that the activities of yeast were not significantly affected by increasing COD and ammonia concentrations.

Variation of pH in one solution (1:1 dilution) during the treatment was measured every 6 hours. It was found that a sharp rise in pH occurred after 6 h. By the time of 12 h, the pH in the solution had increased from the initial value of 4.5 to 7.5 and then remained constant.

Table 4 shows variations of several other parameters before and after batch treatment. It is clear that there was a significant reduction in COD, BOD_5 , total P and reducing sugar but a slight increase in $\text{NH}_4^+\text{-N}$ and protein. The BOD_5 removal of 99% indicates that almost all of the biodegradable

Figure 4. Variations of COD removal under different dilution ratios (wastewater to water) (•••, COD concentration; —, COD removal; ♦, 1:9 dilution; ■, 1:2 dilution; ▲, 1:1 dilution; ○, no dilution).

Table 4. Variations of several parameters before and after batch treatment by yeast (g l^{-1}).

Test items	Wet cell (g)	COD	$\text{NH}_4^+\text{-N}$	TP	Protein	Reducing sugar	BOD_5
Initial	27.3	7.56	6.64	0.01	0.28	1.33	6.7
After treatment	39	1.22	6.68	0.005	0.36	0.05	0.069
Removal rate (%)		84.5		61.5		96.5	99

components in MSG wastewater were decomposed by yeast. The residual COD was perhaps contributed by fermentative intermediates produced in glutamate production, which are normally refractory to microorganisms. Considering the variation of pH before and after treatment, it suggested that the yeast strains mainly utilized small molecular organic acids and reducing sugar but couldn't use protein as their carbon sources. The increase of $\text{NH}_4^+\text{-N}$ indicates that the residual glutamate in MSG wastewater was perhaps used as the main nitrogen source instead of $\text{NH}_4^+\text{-N}$ by yeast.

Bench Scale Treatment

Variations of some important parameters for bench scale treatment system are shown in Figure 5. It is clear that a stable COD removal over 80% was acquired over a period of nearly 2 months in spite of variation of influent COD from 8000 to 25000 mg l^{-1} and the concentration of $\text{NH}_4^+\text{-N}$ from 6326 to 18977 mg l^{-1} . It is interesting that good COD removal performance was maintained even for treating raw wastewater without dilution. The influent pH was adjusted to ca. 3.0 giving an effluent pH of 5.0 to 7.0.

During days 50-57, the percent COD removal was almost unaffected even when the hydraulic retention time (HRT) was shortened from 40 to 30 h (the corresponding organic loading rate of the reactor increased from 10 to 18.7 $\text{kg COD m}^{-3} \text{d}^{-1}$). The yeast treatment system could allow for an organic load rate (OLR) as high as 18.7 $\text{kg m}^{-3}\text{d}^{-1}$, which was

nearly 10 times that of an aerobic membrane bioreactor (1-2 $\text{kg COD m}^{-3} \text{d}^{-1}$) [16].

During the two months' operation of the yeast treatment system under nonsterile condition, the SS of effluent was also monitored every day (the values not shown here). The SS of effluent increased with the increasing of influent COD, and was about 4500 mg l^{-1} when raw wastewater with a COD of 25000 mg l^{-1} was fed. Microscopic analysis showed that most of the cells in the effluents were yeast, indicating that yeast was dominant in the reactor. In other words, ca. 4.5 g yeast cells were obtained by decomposing 20 g of COD. Nourishment analysis indicates that dry yeast cells discharged with effluents contained 55.8% crude protein and 18 kinds of amino acids (values not shown here). These yeast cells could be separated from effluents and be used as an animal forage additive following proper treatment.

The effluents from the yeast reactor still contain nearly 5000 mg l^{-1} COD and a very high concentration of $\text{NH}_4^+\text{-N}$. The residual COD of wastewater could be finally reduced under 500 mg l^{-1} by a combination of activated sludge treatment and coagulation process (Data not shown here). As for high concentration of ammonia in the effluents, conventional biological technologies are not suitable. The process of NH_3 recovery and reuse to the production line using physical-chemical methods is seen as a desirable one. The facts that most COD was removed and the pH increased in the wastewater after treatment by yeast should be helpful to the treatment of $\text{NH}_4^+\text{-N}$.

Figure 5. Variation of COD in bench scale treatment system (◆, influent COD; ■, effluent COD; ▲, COD removal).

CONCLUSIONS

Two yeast isolates identified as *Candida haplophila* and *Rhodotorula glutinis* were isolated from sludge samples contaminated with monosodium glutamate manufacturing wastewater. Their mixture could endure as high as 25% $(\text{NH}_4)_2\text{SO}_4$ when cultivated in an artificial medium and could effectively remove COD of MSG wastewater without being significantly inhibited even when the concentrations of $\text{NH}_4^+\text{-N}$ and free $\text{NH}_3\text{-N}$ reached as high as 18977 and 879 mg l^{-1} , respectively.

In nearly 2 months of bench-scale continuous operation, stable yeast treatment of over 80% COD removal was maintained at a retention time of 30-40 h even when raw wastewater was fed and some important parameters were investigated. The effluents from the yeast reactor contained

large concentrations of SS, ammonia and nearly 5000 mg l^{-1} residual COD. The SS of effluent most of which consisted of yeast cells was on average ca. 4500 mg l^{-1} when raw wastewater was fed and can be considered as animal forage additive. The residual COD in the effluents of the yeast reactor could be further reduced to under 500 mg l^{-1} by applying a combination process of activated sludge treatment and coagulation technology (data will be shown in another paper). Further study is required to remove $\text{NH}_4\text{-N}$ from effluents of the bioreactor.

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