Microbial Protein Enrichment and Treatment of Winery Residue from Fungi and Yeast by Syntrophic Fermentation

Rui Gao, Qiuying He, Yuanyuan Chen, Yuting Duan, Li Xie*

Key Laboratory of Yangtze River Water Environment, Institute of Biofilm Technology, College of Environmental Science and Engineering, Tongji University
E-mail: sally.xieli@tongji.edu.cn

Abstract. Yellow wine lees and rice wastewater are seasonally discharged with high amount of organics. The potential of cultivating Candida utilis and Geotrichum candidum to produce value-added single cell protein (SCP) and simultaneously bio-transform the wastes was investigated. A series of batch experiments were conducted under sterile condition. Co-culture matrix of Candida utilis and Geotrichum candidum resulted in the highest biomass and crude protein yield of 4.2g increased biomass/initial biomass and 68.4%, respectively. Response surface analysis was applied to optimize the fermentation process. The optimal conditions for SCP production with 66.3% of crude protein content were fermentation time of 4 days, solid-to-liquid ratio of 6% (w/v), inoculation proportion of 1:1 (ratio of co-cultures (v/v)), inoculum dose of 18% (v/v), and aeration rate of 1.4 volume of air/volume of reactor per minute. The soluble chemical oxygen demand (SCOD) and the total nitrogen removal efficiency were 78% and 55% respectively. Acetic acid was the main species contributing to the residual SCOD. Furthermore, the contents of essential amino acids closely matched commercial standard for fishmeal and soybean meal, providing high possibility of animal food application.

1. Introduction

Certain amount of wine lees and concentrated wastewater are produced during the wine-making process [1]. These residues contain high organic content resulting in a considerable chemical oxygen demand (COD>50,000 mg/L), and low pH values (pH<5) [2]. In addition, the high content of solid waste and wastewater are produced seasonally, mainly during the harvest period. It is similar with the grape wine and Japanese rice wine which are all seasonally generated, leading to the uneven and variation discharge of wine lees and wastewater. Thus the fluctuated discharge limits the application of conventional biological treatment. For example it was found that installation and operation of anaerobic digester for Japanese rice wine lees treatment were very costly [3]. Currently, the winery wastes from sugarcane ethanol, corn ethanol, and grape winery were utilized and bio-transformed for value-added products like single cell protein (SCP) [4, 5].

SCP as a source of protein can be used for animal feed, appearing as an economically and environmentally friendly alternative. Yeast biomass production as SCP using Candida utilis species has been extensively studied [6,7]. While syntrophic fermentation with fungi and yeast might be critical for SCP yield improvement [8]. The growth of syntrophic microbial was found to be depended on operational conditions, such as substrate, and aeration rates [4, 5]. The transformation of organics were seldom discussed. More work has to be conducted on it. Therefore in this study, Chinese yellow wine lees and its rice wastewater were chosen as the typical winery residue and examined. The residues contain high amount of organics, such as amino acids, lactic acids, and protein, which probably are suitable substrates for SCP production. The main
objective of this study was to evaluate the production and quantity of microbial biomass in mixed cultures of *Candida utilis* and *Geotrichum candidum*. The fermentation process was optimized, and the pollutants transformation and characterization of the obtained SCP were discussed thereafter.

### 2. Methods

#### 2.1. Substrate

The yellow wine lees were collected after pressing and the rice wastewater was collected after rice steeping. Both of them were obtained from Tangsong Wine, Shaoxing, Zhejiang Province, China. The yellow wine lees contained 36.3% crude protein, 46.7% total solid and 45.7% volatile solid. Rice wastewater was acidic with SCOD 55800±283 mg/L, total nitrogen 2620±85 mg N/L, total phosphorus 3338±3 mg/L, suspended solid 34290±630 mg/L. All collected substrate samples were stored in -4°C.

#### 2.2. Fungi and cultivation conditions

Freeze-dried cultures of the yeast *Candida utilis* and fungus *Geotrichum candidum* were obtained from the China General Microbiological Culture Collection Center (CGMCC). The cultures were reactivated in Yeast Peptone Dextrose (YPD) liquid medium and grown on YPD solid medium at 28°C for 3 days. To prepare fungal spore suspension, the fungal spores were harvested and kept in YPD liquid medium at 28°C and 150 rpm. After 20h of cultivation, a spore suspension of 10^7 spores/mL can be inoculated into substrate as fungal inoculum.

Yellow wine lees and rice wastewater were mixed together with a solid-to-liquid ratio of 6% (w/v) to prepare the cultivation substrate. The fungal inoculum was inoculated into a 500mL flask containing 300mL substrate to start the fermentation. Fungal cultivation was conducted at 28°C with nutrient supplementation of peptone. Fungal biomass was harvested after 4d of fermentation.

#### 2.3. Analytical method

The daily fermentation samples were analyzed for SCOD, TN, total sugars, crude protein and amino acids. During fungal cultivation, liquid samples were filtered through 0.45μm filters before analysis. SCOD, TN, NH_4^+-N and TP were analyzed using vial kits from HACH (HACH Company, Loveland, CO, USA). The suspended solids analyses were performed as described in Standard Methods [9]. Crude protein was determined by anthrone colorimetric method and semi-micro Kjeldahl method, and expressed as N×6.25, respectively. The dry weights of fungal biomass samples were measured to calculate the fungal biomass yields defined as g biomass increase/g initial biomass.

### 3. Results and discussion

#### 3.1. Microbial protein yield and protein content of mono and co-culture

The time course of microbial protein production and crude protein content are described in Figure 1 under mono (*Candida utilis, Geotrichum candidum* respectively) and co-culture matrix. As shown, lag phase was observed in the first 3 days fermentation, with slow increase of biomass yield and protein content. The biomass yield and protein content achieved the highest amount at the 4th day, and then remained steady after that. Compared to *Geotrichum candidum, Candida utilis* presented better performance on microbial biomass production, with the yield of 3.68 g increased biomass/initial biomass and protein content of 60.1%. The biomass yield was further improved to 4.21 g increased biomass/initial biomass and protein content to 68.4% in the co-culture matrix, while the biomass production in the mono cultures (*Candida utilis, Geotrichum candidum*) was only 1.07 and 3.68 g increased biomass/initial biomass with protein content of 60.1% and 52.4%, respectively.
The results indicated that the maximum biomass and protein production was achieved by co-culture of *Candida utilis* and *Geotrichum candidum*. These were in agreement with the study of Yao [10], that co-culture system was beneficial for SCP production. In the study, co-culture of three species, *Candida tropicalis*, *Geotrichum candidum* and *Candida utilis* can produce the highest microbial biomass from molasses wastewater. The fungal assimilation of cellulose provided monosaccharide and disaccharide, which probably contributed to better growth of the yeast. Therefore, co-culture matrix of *Candida utilis* and *Geotrichum candidum* and fermentation time of 4 days were selected for inoculation for the following study.

3.2. Optimization of co-culture ratio, inoculum dose and aeration rate by response surface analysis

![Diagram](image.png)

**Figure 1.** Effects of mono and co-culture on biomass yield (a) and crude protein content (b)
The response surface methodology was applied to further optimize the co-culture fermentation process to improve the crude protein content in total fungal biomass. The impact of reaction variables including inoculum dose, co-culture ratio and aeration rate on crude protein content yield are shown in Figure 2 by holding one factor at the zero level and varying another two factors at a time. Figure 2a demonstrated that co-culture ratio and aeration rate were almost independent with respect to the crude protein content and were without interaction effects among each other. It was observed that the crude protein content increased to a maximum of 68.6% with the co-culture ratio of 1:1, aeration rate of 1.48vvm. Figure 2b shows that the decrease of inoculum dose led to the decrease of crude protein content in the range of aeration rate investigated (0.5-2vvm), while it had a greater effect on the crude protein content at lower aeration rate (<1.25vvm). The optimal crude protein content yield was 66.0% when the inoculum dose was 18.65% and aeration rate was 1.42vvm. Similarly from Figure 2c, the decrease of inoculum dose led to the decrease of crude protein content in the range of co-culture ratio investigated (0.3-3), but the effect was more obvious at lower co-culture ratio (<0.3). According to the highest peaks, crude protein content could be as high as 67.9% when the inoculum dose was 16.7% and co-culture ratio was 1:1. It was found that co-culture ratio, inoculum dose and aeration rate as well as interaction between parameters had a positive effect on soluble response.
Figure 2. 3D surface plots and contour plots for crude protein content. a) Effect of co-culture ratio and aeration rate with constant inoculum dose(%), b) Effect of inoculum dose and aeration rate with constant co-culture ratio, c) Effect of inoculum dose and co-culture ratio with constant aeration rate(vvm).

The highest peaks could be observed in each response surface plot which means that the maximum SCP production can be obtained inside the experimental range. The maximum crude protein content was estimated by the optimum conditions were: inoculum dose 18.4%, co-culture ratio 1:1 and aeration rate 1.45vvm, corresponding to the highest crude protein content of 68.7%. In order to confirm the effectiveness of optimal condition obtained by RSM, an additional three experiments were conducted. The crude protein content in the experiment was 66.3%, which was close to the predicted result. These results clearly demonstrated the effectiveness of the model.

3.3. Bioransformation of organics and amino acids in SCP
SCOD and TN are the main indicators for organics in wastes. As shown in Figure 3a, SCOD can be effectively removed in both mono- and co-culture matrixes. It was found that the SCOD was reduced from initial 77200 mg/L to 16880 mg/L after 4 days fermentation in co-culture system, with removal efficiency of 78.1%. Most organics was utilized by yeast and fungi to synthesize SCP, and the residual COD was found to be short chain volatile fatty acids, mainly acetate in this study (data not shown).

The performance of TN removal by three culture systems are compared in Figure 3b. The TN removal rate was highest in Geotrichum candidum mono-culture, which displayed 67.0% removal efficiency. In co-culture fermentation system, the TN removal efficiency was the lowest, which was different from the COD removal performance. The mechanisms of COD and TN degradation by three culture system may be different, which can be further explored in the future.
Fish meal was the most cost-effective animal protein source and as its probable substitute, soybean meal was studied recently [11]. Both contained essential amino acids, among which lysine and methionine are limiting ones for all animals. The composition of amino acids in SCP obtained by fermentation in this study was examined and compared with fish meal and soybean meal. According to the tables of feed composition and nutritive values in China[12], although the compositions of lysine produced in this study(2.20%) were slightly lower than that of fish meal(3.93%)and soybean meal(3.38%), SCP contained more isoleucine (4.12%) and methionine (1.47%), which enabled it to be an efficient nutrition supplement. In conclusion, SCP can be considered as an economic chance for animal food industry by co-feeding with fish meal.

Figure 3. Removal efficiency of COD and TN in the substrate

4. Conclusions
The feasibility of syntrophic fermentation from winery waste for both SCP production and waste recycling was evaluated. Co-culture matrix of Candida utilis and Geotrichum candidum, combined with optimizing conditions obtained better results on biomass production and protein content compared to mono-culture. 78.1% SCOD was removed and the residue organics were short chain fatty acids and acetate was the main species. Higher amount of methionine than that of soybean meal was observed in the fungal biomass, which ensures better growth of animals. Furthermore, co-feeding the SCP with commercial animal feedstock can improve the amount of essential amino acid and partly offset the total cost.
5. References

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