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Exploration on Mixed Cultivation of *Lactobacillus*, *Yeast* and *Bacillus subtilis*

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Abstract: To study on mixed fermentation of probiotics, bacterial cultivation was carried out firstly. It was showed that the optimal pH values for growth of *Lactobacillus*, *Yeast* and *Bacillus subtilis* were 6.5, 6.0 and 7.0, respectively. And the suitable temperature for all of them was about 31°C. Then under selective conditions pH 6.0, 31°C and 160 r/min, three probiotics were cultivated by taking *Lactobacillus* as main bacteria and the others as auxiliaries. It was found that the desired bacterial number ratio of *Lactobacillus*: *Yeas*t: *Bacillus* for mixed cultivation was 1:2:2 and the OD value of mixed bacterial solution achieved at 2.27 after three days.

Keywords: Mixed Cultivation, *Lactobacillus*, *Yeast*, *Bacillus subtilis*

1. Introduction

Probiotics are beneficial to the regulation of animal intestinal microecological balance system and the restoration of water environment, thus they can be used as feed additives and microecological bacteria agent for water environment improvement [1]. Commercial probiotics usually were consisted of one bacteria prepared by single strain fermentation, and the practical effect of them are limited. Proved with a mixture fermentation of various compatible strains can be carried out, and then a mixture of probiotic bacteria agent will be gotten, might having better physiological effect. Comparing with the strain-separated fermentation, the mixed method can not only avoid waste of resources but also allow bacteria interactions in fermentation process [2]. Common probiotics are there *Lactobacillus*, *Yeast* and *Bacillus subtilis*, etc. [3]. *Lactobacillus* is widely used in areas such as pharmaceutical, food, light industry and animal husbandry [4]. *Lactobacillus* can colonize in the body of host and inhibit proliferation of disease-causing bacteria. They can maintain the ecological balance and enhance the body's immune function, and also can live well in the aquatic environment and play a role of dominant bacterial community [5]. Metabolism of *Lactobacillus* produces enzymes, organic acids and bacterial surface composition, etc., which can promote the development of the body's nutritional state, toxic reaction and immune response, tumorigenesis, aging process and stress reaction, towards good effect [6]. *Yeast* itself rich in nutrition contains a large number of amino acids and a variety of digestive enzymes, which can be used as a feed and drug [7]. *Bacillus subtilis* can produce high activity of digestive enzymes and antibacterial activity protein, which as beneficial bacteria in human and animal intestinal system can biodegrade and transform organic matter, and directly use of nitrate and nitrite [8]. Based on the previously mixed fermentation of several probiotics [9], this paper by cultivating single strain firstly determines the optimum culture conditions of *Lactobacillus*, *Yeast* and *Bacillus subtilis*, respectively. And then they were mixed and cultured by taking *Lactobacillus* as main bacteria and the other two as auxiliary bacteria.

2. Materials and Methods

2.1. Main Apparatus

Main apparatus were constant temperature incubator (HPS - 400, Harbin), vertical high pressure steam sterilization pot (HX03L250, Shanghai), super clean workbench (SW-CJ-1FD, Shanghai), constant temperature shaking incubator (HZQ, Harbin), ultraviolet visible spectrophotometer (Alpha-1500, Shanghai), and microscopy (BA410, Shanghai).

2.2. Materials

Lactobacillus was presented by BEIJIAMEI biotechnology research and development company, *Yeast* was provided by ANGEL *YEAST CO*., LTD, *Bacillus subtilis* was offered by Biological Synthesis and Biological Medicine Research and Development Center of Tianjin Agricultural University. Culture medium compositions including *Yeast* extract powder, beef extract, lactose, glucose, peptone, agar, and phosphate, etc., were bought in the market. To refer literature [10], The liquid (solid) medium of *Lactobacillus* was consisted of *Yeast* extract powder, beef extract, lactose, glucose, K_2HPO_4, polysorbate 80, sodium acetate, agar, tomato juice and cold water in a mass ratio of 5:10:20:2:2:1:5:0(15):50:1000. The liquid (solid) medium of *Yeast* contented sucrose, K_2HPO_4, $MgSO_4$, $(NH_4)_2SO_4$, agar and cold water. The mass ratio of them was 5:5:2:3:0(15):1000. The liquid (solid) medium of *Bacillus subtilis* included peptone, beef extract, NaCl, agar and cold water, which have a mass ratio of 10:3:5:0(15):1000. Method for preparing tomato juice was to keep fresh chopped tomatoes at 4°C for 8 to 12 h, and then filter and undergo high pressure sterilization.

2.3. Preparation of Bacterial Solution

Lactobacillus: Under aseptic conditions, 1 mL *Lactobacillus* was dropped into a 250 mL conical flask with 100 mL sterilized liquid medium. And the flask was plugged and sealed with the sealing membrane. The bacteria was cultured for 24 h at 37°C and 160 r/min. After repeating the above performance twice, the last culture solution was kept at low temperature and used to continue to subculture.

Yeast: Under aseptic conditions, 2 g *Yeast* was added into 100 mL sterile water for dissolution and kept for 6 h at 28°C and 160 r/min. And then 1 mL of Yeast solution was taken out to add in a 250 mL conical flask with 100 mL sterilized liquid medium. Follow the flask was sealed and cultured at 28°C and 160 r/min for 24 h. Again taking 1 mL culture solution continuously repeated culturing twice. The last culture solution was kept at low temperature and used to continue to subculture.

Bacillus subtilis: Under aseptic conditions, one ring of *Bacillus subtilis* strain was taken from inclined medium and put in a 250 mL conical flask with 100 mL sterilized liquid medium. Then the flask was sealed and cultured at 37°C and 160 r/min for 24 h. Taking 1 mL culture solution processed subculture twice. The last culture solution was kept at low temperature and used to continue to subculture.

2.4. Culturing Under Different pH and Temperature

After sterilization, liquid medium was adjusted with 1 mol/L HCl and 1 mol/L NaOH to the demanded pH values. Then the corresponding bacterial strain was added and cultured at 37°C. Every 6 h, the OD values of bacteria liquid were taken out and detected by taking the original culture medium as reference for zero setting. Thereafter culturing every bacterium for 6 h at the optimal pH, 160 r/min and different temperature measured the OD value of bacterial liquid.

2.5. Counting and Mixed Culturing

Bacterial strain number of bacteria liquid was calculated by the plate count method [11]. Bacterial liquid was diluted by 10^3, 10^4, 10^5, 10^6 and 10^7 times, respectively. And 0.1 mL diluent was taken to make three parallel coating experiments for culturing, respectively. After that, selecting the fitting pH and temperature conditions for growth of three kinds of bacteria mixed and cultured them according to various bacterial number proportion of them. OD values of mixed bacterial solution were detected before and after cultured for 3 days, respectively.

3. Results and Discussion

3.1. Effect of pH on Bacterial Growth

Refer to related researches [10], liquid mediums with various pH values was used to culture three bacteria and the OD values of bacterial solutions were detected every 6 h. The detected results were showed by the growth curves of them at 37°C and 160 r/min (Figure 1). At pH 6.5, *Lactobacillus* grew in the biggest rate and quickly achieved at the maximal OD value. At pH 5.5 and 6.0, the bacteria behaved sluggish in earlier stage, developed exponentially in medium term, and grew stably in later period. The pH 4.5 and 5.0 were not fit the bacteria for growth (Figure 1. a). *Yeast* was cultured at 28°C and 160 r/min, and reproduced in faster rate at pH 6.0, which also exhibited growth sluggish firstly, quick successively and steady lately, respectively. Though the growth trend line of *Yeast* at pH 5.5 was similar as at pH 6.0, but the maximal OD value was smaller than at pH 6.0. And other pH values obviously were not advantageous of growth and endowed Yeast with slow growth (Figure 1. b). As to *Bacillus subtilis*, it was cultured at 37°C and 160 r/min and the growth curves were showed in Figure 1. c. This bacteria reproduced well at pH 6.0-8.0.

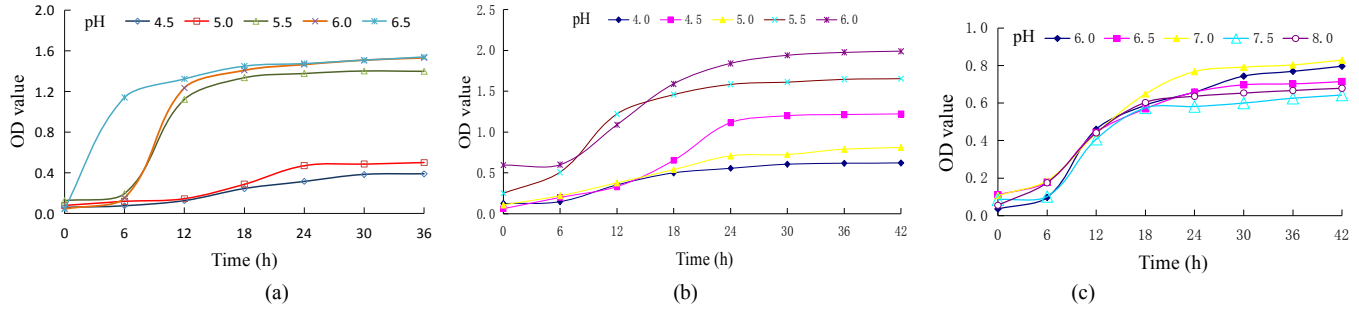

Figure 1. *Growth curves of three bacteria: (a) Lactobacillus; (b) Yeast; (c) Bacillus subtilis.*

3.2. Effect of Temperature on Bacterial Growth

At the optimal pH values of three bacteria, they were cultured for 6 h at 28-32°C and the OD values of bacterial solutions were seen in Figure 2. It was showed that the fastest reproducing for *Lactobacillus* was at 31°C, and *Yeast* growing altered little along with temperature changing from 28°C to 32°C, i.e., only weak effect of temperature on growth of *Yeast*. The growth rate of *Bacillus subtilis* increased with the rise of temperature and tended to a stable growth level when reaching a certain high temperature.

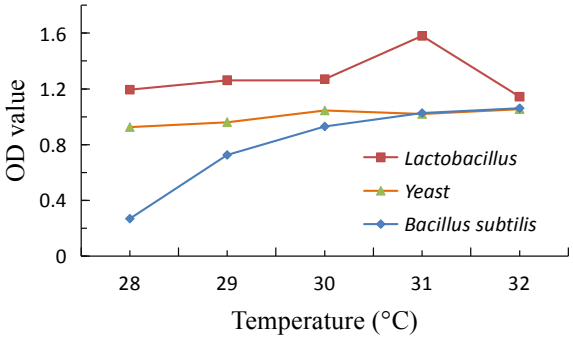

Figure 2. *Comparison of OD values for three bacteria at different temperature.*

3.3. Mixed Culture of Three Bacteria

According to the relationship between three kinds of bacteria growth and both of temperature and pH, they had bigger growth rate at 31°C and pH 6.0. Thus the temperature and the pH value were selected to culture the mixed strains at 160 r/min. Under this condition, it was especially beneficial to the growth of *Lactobacillus*, which could reproduce by taking advantage of sugar produced from *Yeast* and *Bacillus subtilis* [2]. Namely, *Yeast* and *Bacillus subtilis* could promote growth of *Lactobacillus*. Therefore, *Yeast* and *Bacillus subtilis* were employed as auxiliary bacteria to help the growth of *Lactobacillus*. Using dilution coated plate count method [11], concentrations of *Lactobacillus*, *Yeast* and *Bacillus subtilis* liquids were analyzed to be 4.6×10^7, 1.0×10^8 and 1.95×10^7 cfu/mL, respectively (Table 1). In the basis of these concentrations, it was to design number proportion of three bacteria for mixing them. *Lactobacillus*, *Yeast* and *Bacillus subtilis* were mixed and cultured according to bacterial number ratio of 1:1:1, 1:1:2, 1:2:2, 1:2:3 and 2:2:3, respectively. The results of mixed culture were seen in Figure 3. It was shown that, under the selected condition, the experimental group of mixed bacteria reproducing better was one with a bacterial number proportion of 1:2:2 and the OD value was 2.27.

Table 1. *Analysis on concentration of bacterial solutions.*

Strains	Dilution ratio	Flat colonies (cfu)	Colony average (cfu)	Microbial concentration (cfu/mL)
Lactobacillus	10^6	44/48/46	46	4.6×10^7
Yeast	10^6	95/100/105	100	1.0×10^8
Bacillus subtilis	10^5	201/186/198	195	1.95×10^7

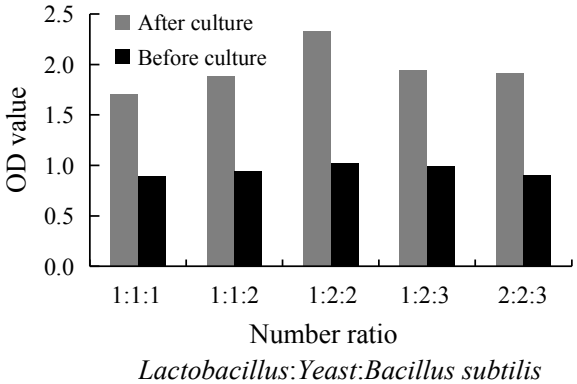

Figure 3. *OD values of mixed bacteria before and after cultured for 3 d.*

4. Conclusions

It was found that the most suitable conditions for *Lactobacillus* growing were at pH 5.5-6 and 31°C. *Yeast* had normal growth curves at pH 6.0 and 28-32°C, which was fit for *Yeast* breeding. *Bacillus subtilis* had culture curves in accordance with bacterial growth rule at pH 6.0-8.0, and the best suitable temperature for its growth was over 32°C. When they were mixed and cultured, the desired bacterial number ratio of *Lactobacillus*: *Yeast*: *Bacillus subtilis* was 1:2:2. In this ratio, they were cultured for 3 d at pH 6.0, 31°C and 160 r/min. The OD value of mixed bacteria liquid was 2.27.

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