# Fermentation of Antarctic Red Yeast AN5 and Its Effect on Sea Cucumber Body Composition

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**Abstract**

Red yeast is a common type of marine yeast and is often used as a probiotic in aquaculture. In this experiment, the red yeast AN5 isolated from sea ice was studied in the 22nd Antarctic survey, and the fermentation growth conditions of yeast were optimized. The optimal fermentation conditions for AN5 were as follows: 2% molasses, 0.5% yeast powder, 50 mL medium in 250 mL flask, initial pH=3.0, temperature 20°C, and cultivation for 4 days. Under optimized conditions, the number of yeast cells can reach $5.81\times10^7$ cells· $ml^{-1}$. Component analysis of AN5 showed that the contents of water, crude protein, total sugar, and crude fat were 69.15%, 42.00%, 32.08%, and 0.39%, respectively. Juvenile sea cucumbers were fed a diet containing 0.3 g polar yeast per cubic meter water volume. After two months of culture, compared with the control, the water and fat content of the juveniles did not change substantially, while the protein and polysaccharide content increased significantly. Therefore, the polar red yeast can be fermented in large quantities and used as a microecological preparation in the cultivation of sea cucumber.

**Keywords**

Marine yeast; Antarctic red yeast; Culture optimization; Juvenile sea cucumber; Microecologics.

## Introduction

Red yeast is a dominant species of marine microorganisms, which are widely distributed in various sea areas, north and south poles and deep seas. The ocean is a unique environment with high pressure (deep sea), low nutrition, low light, and large changes in temperature and salinity, so that the yeast cells in the metabolism and products of the yeast are different from the yeast living in the terrestrial environment. Marine red yeast cells are rich in active metabolites such as proteins, carotenoids, polysaccharides, astaxanthin, unsaturated fatty acids, essential amino acids, digestive enzymes, vitamins, etc., which have high nutritional value and activity. It could be used not only for the production of carotenoids, single-cell proteins and biodiesel, but also for micro-ecological preparations, purification of water. And it is widely used in medicine, food, chemical, agricultural and environmental protection (Loque et al., 2010).

Marine red yeast is about 4-6 μm in size. Being rich in nutrients, good in palatability, not easy to precipitate, it has good salt tolerance. At the same time, it has the advantages of short culture period, strong adaptability and low cost. It is very suitable as an open bait for larvae of marine animals and a supplementary bait for larval period. It can significantly improve the

survival rate of aquatic animal seedlings, improve the utilization rate of feed, enhance the immunity of animals, and purify water bodies. It is an excellent additive for ecological breeding. As a cultured microecologics, marine red yeast has been widely used in aquaculture and has been extended to more than ten cities. Compared with the general marine environment, the Antarctic has extreme environmental characteristics such as low temperature, high salt, low light, oligotrophic and strong radiation (Yang et al., 2011). It enriches low-temperature microbial resources including yeast, and it is capable of producing unique metabolites. Therefore, this study used Rhodotorula mucilaginosa AN5 as the experimental object to study the optimal growth conditions for fermentation culture and preliminarily discuss on the possibility of application as a micro-ecological preparation for aquaculture.

## Materials and Methods

### The Experimental Strains

The marine polar yeast Rhodotorula mucilaginosa AN5 was isolated from sea ice samples collected from the 22$^{nd}$ Antarctic scientific expedition.

### Yeast Medium and Culture

YPD Culture medium: Glucose 2.0 g, peptone 2.0 g, yeast powder 1.0 g, pH=6.0, seawater 100 mL, sterilized for 30 min at 0.1 MPa. The yeast activated for 4 days was inoculated in liquid YPD medium at 120 r·min$^{-1}$ and shaken cultivated at 20 °C.

### Yeast Density Measurement

Microscopic counting method was used.

### The Cultivation of Sea Cucumber

The sea cucumber used in the experiment was a seedling of sea cucumber, which was about 0.5-0.8 cm long. The juvenile sea cucumbers were randomly divided into 6 glass jars for artificial breeding (3 for the control group and 3 for the experimental group), and the same amount of artificial compound feed was fed regularly every day. The experimental group was fed 0.3 g of red yeast every day, and changed half water every 2 days. After 2 months, sea cucumbers were collected, and the surface moisture was absorbed and stored at −20 °C (Dhaliwal and Chandra, 2016).

### Composition Determination Method

The moisture content is determined by the national standard GB5009.3-2010 direct drying method. Protein content is determined according to national standard GB5009.5-2010 Kjeldahl method. The crude fat content was determined by soxhlet extraction method.

### Data Analysis

The test data is the average of 3 biological replicates, expressed as mean ± standard error. Analysis of variance and difference significance were performed using one-way ANOVA in software SPSS 15.0. Among which, $P < 0.05$ was considered significant, and $P < 0.01$ was extremely significant difference.

## Results and Analysis

### Effect of Culture Conditions on Yeast Fermentation Growth

(i) Effect of carbon source on yeast growth

The glucose in YPD medium was replaced by 2% corn starch, sucrose, molasses, cornmeal, bean noodles and millet noodles, the other components and culture conditions were unchanged, and the cell density was determined by microscopic counting after 4 days (Figure 1).

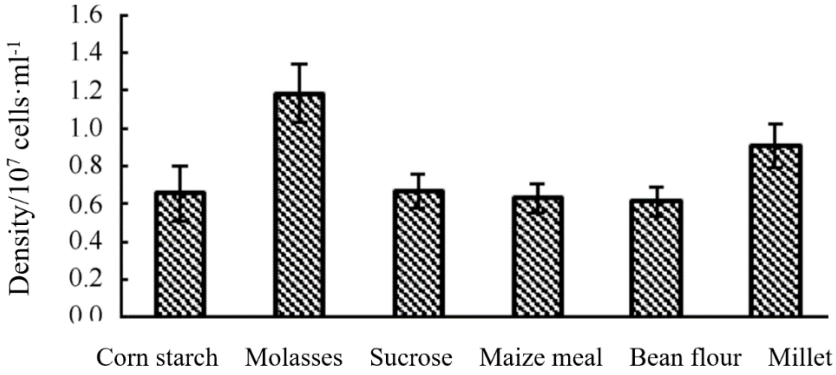


Figure1: Effect of different carbon sources on Antarctic yeast AN5 growth.

Among the six carbon source materials, when the molasses was used as the carbon source for fermentation, the yeast cell density reached a maximum of $1.19\times10^7$ cells·mL$^{-1}$, followed by the millet surface of $0.91\times10^7$ cells·mL$^{-1}$, corn starch, cornmeal, bean noodles and sucrose were about $0.65\times10^7$ cells·mL$^{-1}$, which was significantly smaller than the first two. Molasses contain nutrients, vitamins and trace elements necessary for the growth of marine red yeast.

The red yeast was cultured at a concentration of 1.0%, 2.0%, 3.0%, and 4.0%, respectively. The cell density of the yeast is shown in Figure 2.

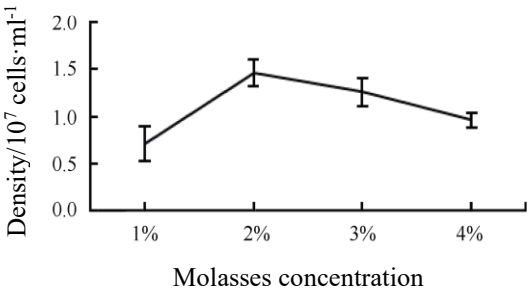


Figure 2: Effect of different molasses concentration on Antarctic yeast AN5 growth.

The results showed that the yeast cell density in the 2% molasses fermentation broth was $1.46\times10^7$ cells·mL$^{-1}$, which was significantly higher than other concentrations, which determined that 2% molasses was the best carbon source for yeast culture.

(ii) Effect of nitrogen source on yeast fermentation

This experiment used the above optimized 2% molasses as a carbon source, liquid corn syrup, solid corn syrup, molasses, cornmeal, yeast powder, soybean cake powder, peptone, soy peptone, ammonium sulfate, potassium nitrate as nitrogen source respectively. After 4 days of fermentation, the yeast cell density was measured (Figure 3).

Among the 10 nitrogen sources, the effect on the growth of AN5 was yeast powder > soy peptone > peptone > corn gluten > solid corn syrup > potassium nitrate > liquid corn syrup > molasses > soybean cake powder > ammonium sulfate. Among the eight organic nitrogen sources, when the yeast powder was used as the nitrogen source for fermentation, the yeast cell density reached a maximum of $1.3\times10^7$ cells·mL$^{-1}$.

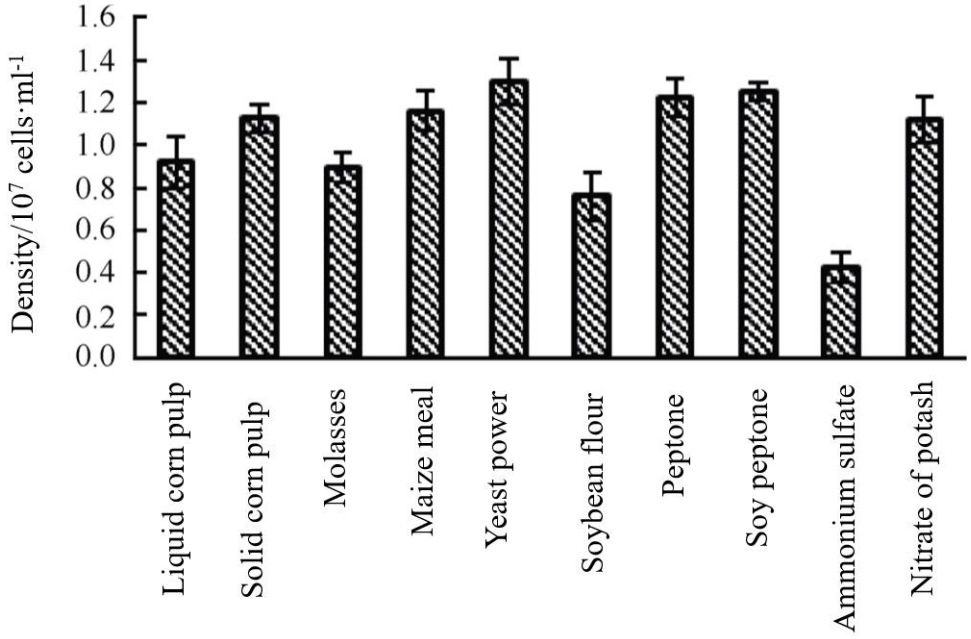


Figure 3: Effect of different nitrogen sources on Antarctic yeast AN5 growth.

Among the two inorganic nitrogen sources, the effect of nitrate nitrogen is much higher than that of ammonium nitrogen. After screening for the best nitrogen source, yeast was cultured at 0.25%, 0.50%, 0.75%, and 1.0% yeast powder concentrations (Figure 4).

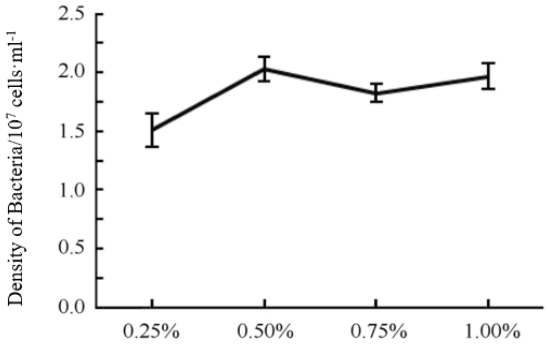


Figure 4: Effect of different yeast powder concentration on Antarctic yeast AN5 growth.

As can be seen from the figure, the yeast cell density in the fermentation broth can reach $2.03\times10^7$ cells·mL$^{-1}$ when fermented with yeast powder at a concentration of 0.5%. The yeast cell density was essentially unchanged as the yeast powder concentration was increased. Considering the density and cost of fermentation, 0.5% yeast powder was chosen as the optimal nitrogen source for yeast culture. (iii) Effect of initial pH on yeast growth (Zaky et al., 2016).

Using the above optimized carbon and nitrogen source as the medium, the initial pH was adjusted to pH values of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, respectively, and the density of the cells was measured after 4 days of culture (Figure 5).

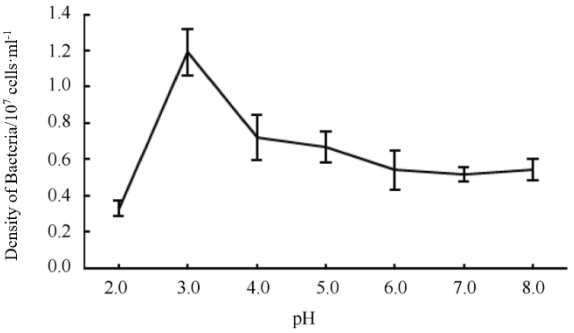


Figure 5: Effect of different initial pH on Antarctic yeast AN5 growth.

When pH=3.0, the yeast cell density in the fermentation broth was the largest, $1.19\times10^7$ cells·mL$^{-1}$. When pH=2.0, the cell density decreased rapidly, reaching the lowest density value of $0.32\times10^7$ cells·mL$^{-}$

[1]. When pH>3, the cell density became smaller with the increase of pH, but the decrease was slow. The density was still $0.54\times10^7$ cells·mL$^{-1}$ at pH=8.0. It can be seen that the optimum initial pH of marine yeast cell culture is 3.0, which is significantly lower than that of normal temperature yeast with an optimum pH of 4.0-5.0.

(iv) Effect of temperature on yeast growth

The yeast was cultured at 10 ° C, 15 ° C, 20 ° C, 25 ° C and 30 ° C with the optimized carbon and nitrogen sources as the medium. The yeast cell density after 4 days is shown in Figure 6.

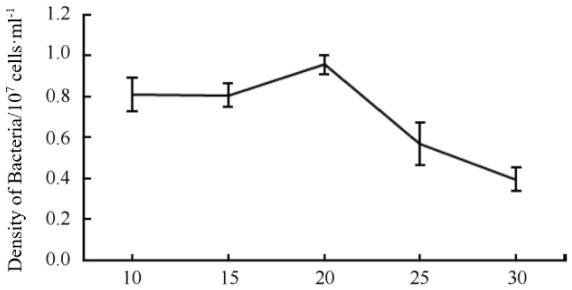


Figure 6: Effect of different temperature on Antarctic yeast AN5 growth.

As can be seen from the figure, when the temperature was raised from 10 ° C to 20 ° C, the cell density reached a maximum of $0.96 \times 10^7$ cells · mL $^{-1}$. Thereafter, as the temperature continued to rise, the yeast density dropped significantly. Therefore, the optimal temperature for the growth of AN5 strain was 20 °C, which should be controlled at this temperature during fermentation (Chi et al., 2016). The yeast used in the experiment is marine polar yeast, generally lower than the optimum growth temperature of normal temperature yeast 25-28 °C.

(v) Effect of liquid volume on yeast growth

The medium and yeast seed solution were added to a 250 mL flask to make a final volume of 30, 50, 70, 90, 110, 130, 150 mL. (Figure 7).

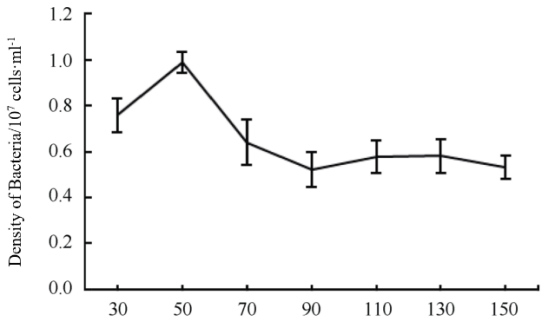


Figure 7: Effect of different liquid volume on Antarctic yeast AN5 growth.

When the liquid volume was 50 mL, the yeast cell density reached a maximum of $0.99\times10^7$ cells·mL$^{-1}$. When the liquid volume was 30 mL and 70 mL, the cell density was significantly reduced.

The effect of different liquid loading on yeast growth is reflected in two aspects: (1) The larger the liquid volume, the more the nutrient content is, which provides material guarantee for the growth of yeast. (2) The larger the liquid volume, the smaller the remaining space, and the less air in the triangular flask.

(vi) Effect of rotation speed on yeast growth

The optimized carbon and nitrogen source were used as the medium, the liquid volume was 50 mL/250 mL, and the shaking speed of the shaker was set to 80, 100, 120, 140, 160 and 180 r·min$^{-1}$, respectively. (Figure 8).

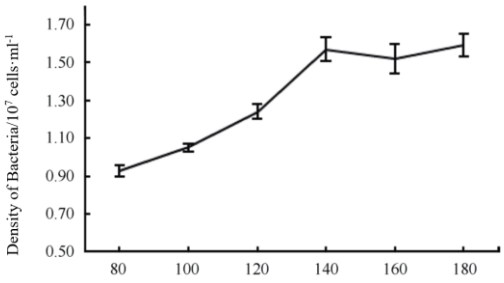


Figure 8: Effect of different rotation rate on Antarctic yeast AN5 growth.

It can be seen from Figure 8 that as the rotation speed of the shaker increases, the yeast fermentation density increases. From $0.93\times10^7$ cells ·mL$^{-1}$ at 80 r·min$^{-1}$ to $1.59\times10^7$ cells·mL$^{-1}$ at 140 r·min$^{-1}$, it increases 71%. Since then, with the increase of the rotational speed, the cell density has almost no change, so the optimal shake flask speed in this experiment is 140 r·min$^{-1}$. The factors affecting the fermentation density of the bacteria in the shake flask culture are basically the same. When the oxygen content reaches a certain limit, the density of yeast does not increase. After optimization of the above conditions, the optimal fermentation conditions of the red yeast AN5 are obtained. At this time, the yeast density reached $5.81\times10^7$ cells·mL$^{-1}$, but lower than other normal temperature red yeasts (Duarte et al., 2013).

**The Determination of Main Components in**

**Rhodotorula**

The red yeast AN5 was fermented in a large amount under optimized culture conditions, and the obtained cells were subjected to measurement of water and main nutrients. The average water content of Rhodotorula glutinis AN5 was determined to be 69.15%. The main nutrient composition determination results were shown in Table 1.

Table 1: Main nutrient composition in several red yeasts Rhodotorula.

| Saccharomycopsis | Protein content | Fat content | Contents of total sugar |
|---|---|---|---|
| R. mucilaginosa AN5 | 42.00%±2.93% | 0.39% | 32.08% |
| R. mucilaginosa | 49.2% | 1.5% | 22.3% |
| R. paludigenum | 42.01% | 3.09% | 29.5% |

It can be seen from the table that the crude protein content in dry yeast is about 42.00%, which is similar to the protein content in normal temperature red yeast, which is in line with the protein requirement of biological animals in aquaculture. Total sugar is the main source of energy in the organism. The total sugar content of yeast AN5 is 32.08%, which is higher than that of normal temperature yeast. Adequate sugar can be used to consume energy from living organisms and play an important role in aquaculture.

**Effects of Red Yeast on Water and Main Nutrients of Juvenile Sea Cucumbers**

The sea cucumber seedlings were fed with 0.3 g of fresh yeast per day, and after 2 months, the water and main nutrient contents of the sea cucumber were measured (Ban and Lane, 2016). Normally cultured sea cucumbers had a water content of 92.02%, sea cucumbers fed with red yeast owned water content of 91.83%, and there was no significant difference in water content. After feeding red yeast, the dry weight of protein increased from 35.84% to 43.19%, and the content increased significantly. The fat content changed from 7.77% to 7.95%, with no significant change. However, the content of polysaccharide in sea cucumber added with red yeast was 6.75% dry weight, which was 23.63% higher than the control group of 5.46%.

## Discussions

**The fermentation production of yeast**

Large-scale fermentation production of yeast is the premise and basis for the application of red yeast. Scientists have found that different culture conditions and fermentation processes affect the biomass of marine red yeast (Yang et al., 2013). By adjusting the type and amount of carbon source, nitrogen source, inorganic salt, growth factor and other substances in the medium, the conditions such as pH value, inoculum amount, liquid volume, and aeration amount during the culture process are changed, which can significantly increase the yield of yeast (Ribeiro et al., 2011).

The carbon and nitrogen sources used in this experiment are all industrial fermentation raw materials, which are cheap and easy to obtain. After optimization of the fermentation process, the yeast cell density increased by 6.21 times. The biomass of yeast fermentation at room temperature was reached, which laid a foundation for large-scale fermentation production of yeast cells (Subramanian et al., 2014).

However, this experiment has only screened a single carbon and nitrogen source. Studies have shown that the combination of multiple nutrients is more conducive to yeast growth. This is the direction of the next step of research. In addition, the study only optimizes the fermentation with biomass as the index (Fu et al., 2015). The influence of fermentation conditions on the main functional active factors needs further study to improve the nutritional value of the yeast. It is worth mentioning that the optimum fermentation temperature of the red yeast is 20 ° C, and the optimum initial pH is 3.0. Low temperature and low pH culture can inhibit the growth of bacteria and reduce pollution.

**Application Potential of Red Yeast as a Feed Additive**

Red yeast is a single-celled microorganism widely found in the ocean. The complex living environment creates unique metabolites and active substances in the body (Vongsvivut et al., 2013). The yeast itself has no toxic side effects and high safety, and has wide applications in fruit preservation, livestock and poultry farming. Marine red yeast and its fermentation products are rich in protein, sugar, astaxanthin, vitamins, minerals, etc., they contain more essential amino acids, and have comprehensive nutrition. It is a micro-ecological preparation that integrates nutrition and health care. These special nutrients make up for the nutritional deficiencies of conventional baits (or additives), improve the resistance of aquatic animals, reduce the use of antibiotics, and improve the quality of aquatic products (Babu et al., 2013).

The application of Rhodotorula as a microbial bait in the field of aquatic seed breeding began in the 1990s, and scientists had done a lot of research. In this study, the content of protein and polysaccharide of Radix isatidis is high. As a microecological preparation, it can significantly increase the content of protein and polysaccharide in sea cucumber (Penglase et al., 2011). After sea cucumber is fed to yeast, it can survive in juvenile ginseng. In vivo superoxide dismutase, polyphenol oxidase, catalase, acid phosphatase, alkaline phosphatase, lysozyme, amylase, cellulase, alginate, nitric oxide synthase, trypsin, fat enzyme activity such as enzymes is significantly increased. It promotes the digestion and absorption of bait, and increases the content of main nutrients such as protein and polysaccharide in sea cucumber, which has obvious promotion effect on growth. At the same time, red yeast provides nutrients directly to juveniles as a nutrient-based biological bait, which can improve the metamorphosis rate and survival rate of sea cucumber larvae.

In addition, yeast has a high nitrification capacity, which can effectively remove nitrite from sea cucumber culture wastewater and improve aquaculture water quality (Wang et al., 2016). These studies have laid the theoretical foundation for the application of yeast in sea cucumber culture. Antarctic red yeast is easy to be fermented on a large scale, and has rich nutritional value. It has the potential as a biological bait organism and is a good feed material. It can be used as a substitute for protein sources such as fishmeal, with good market prospects and development potential (Amagata et al., 2012).

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