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# Breeding of high ergosterol-producing yeast strains

X He, W Huai, C Tie, Y Liu and B Zhang[1]

*Department of Microbial Molecular Genetics and Breeding, Institute of Microbiology, Academia Sinica, Beijing 100080, China*

**High ergosterol-producing yeast strains YEH-28 and YEH-56 were constructed by hybridization of two haploids with opposite mating types from different species. The fermentation conditions of hybrid strain YEH-56 were studied. The highest level of ergosterol was obtained in 30 h at 28°C and 200 rpm, when 60 ml of culture in 250-ml shake flasks was grown in fermentation medium that consisted of (per liter): 100 g glucose; 10 g peptone and 10 g yeast extract. The initial pH was adjusted to 6.0. The optimal inoculum volume was 10% (v/v). Under optimal conditions, the yield of ergosterol of YEH-28 strain is 1.96 and 1.56 times that of the parental strains YE39 and YE244, whereas that of YEH-56 is 1.98 and 1.57 times that of the parental strains YE39 and YE244, respectively. Analysis of genetic stability showed that hybrid strains YEH-28 and YEH-56 are stable genetically.** *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 39–44.

**Keywords:** yeast; hybridization; breeding; ergosterol; biomass

## Introduction

Ergosterol, a precursor of vitamin $D_2$, can be transformed into vitamin $D_2$ by ultraviolet irradiation. Vitamin $D_2$ has important functions in promoting the body to absorb $Ca^{2+}$, $PO_4^{3-}$, and in preventing rickets and osteoporosis. Ergosterol is also the main precursor of cortisone and the hormone progesterone and an additive of fodder to increase the laying and hatching rates of fowls.

Ergosterol formation occurs in many organisms. Ergosterol production by species of *Aspergillus* and *Penicillium* was studied in detail. Some ergosterol-producing strains and their partial biosynthetic pathway have been investigated. Most of the key genes involved in the pathway have been cloned and expressed [1–7,9,12]. As a source of ergosterol, yeasts have gradually received more attention, especially the genus *Saccharomyces*. Although yeast strain natural selection and some factors affecting ergosterol production have been studied [1,3,13–15], the contradiction between biomass and ergosterol content in wild strains, where high biomass is accompanied by low ergosterol content, and *vice versa*, has not been solved. Construction of good strains by genetic technology can partially solve such problems. This paper describes breeding of high ergosterol-producing yeast strains by hybridization and determination of optimal conditions for fermentation.

## Materials and methods

### Strains

The 230 primary yeast strains from different genera and species are stored in our laboratory, designated as YE1 to YE230. *S. cerevisiae* ZF-5-8(a) and ZW-21($\alpha$) are used as the standard mating-type strains and also are stored in our laboratory.

### Media and culture conditions

Test strains were cultured for 24 h on YEPD slants containing (per liter): 10 g yeast extract, 20 g peptone, 20 g glucose and 12 g agar. Seed cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml YEPD liquid medium. They were incubated for 16 h at 28°C on a rotary shaker (200 rpm). For ergosterol production, 50 ml of fermentation medium in a 250-ml Erlenmeyer flask was inoculated with 10% (v/v) of the seed culture and grown for 30 h on a rotary shaker (200 rpm). For sporulation, the strain was inoculated in McClary medium containing (per liter): 10 g potassium acetate, 2.5 g yeast extract and 1 g glucose, and incubated for 3–5 days at 25°C on a rotary shaker. Isolation and characterization of auxotrophic mutants and hybrids were conducted on yeast nitrogen base (YNB) medium and YNB selective medium [10,15].

### Hybridization

Diploid cells from a YEPD slant were transferred to 2-ml McClary liquid medium and incubated for 3–5 days at 25°C on a rotary shaker. Sporulation was monitored by light microscopy. The sporulated culture was harvested by centrifugation. Cells were resuspended in 5 ml of sterile $H_2O$ and held in a water bath for 15 min at 58°C to kill vegetative cells. $\beta$-glucuronidase ($\beta$-D-glucuronide glucuronosohydrolase; EC 3.2.1.31) (1000 units) was added to the suspension and incubated for 1 h at 37°C on a rotary shaker. The digestion of asci walls was checked by light microscopy. The spore suspension was spread on a YEPD plate and incubated until single colonies were well developed. Colonies were transferred on McClary medium again by replica-plating from the YEPD plate and incubated for 3–5 days at 25°C. Possible haploids were obtained by choosing colonies that were unable to sporulate. They were transferred to a YEPD plate and mixed with the standard mating-type strains ZF-5-8(a) and ZW-21($\alpha$), respectively.

Correspondence: X He, Department of Microbial Molecular Genetics and Breeding, Institute of Microbiology, Academia Sinica, Bei Yi Tiao No. 13 Zhongguancun, Haidian, Beijing 100080, China
[1]Also corresponding author.
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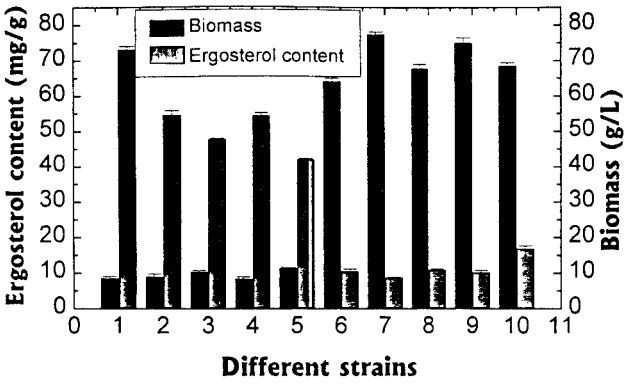


**Figure 1** Comparison of the biomass and ergosterol content of different yeast strains. (1) *S. kluyveri* YE39; (2) *S. cerevisiae* YE84; (3) *Schizosaccharomyces pombe* YE210; (4) *S. carlsbergensis* YE214; (5) *S. cerevisiae* YE226; (6) *S. rouxii* YE104; (7) *Hansenula anomala* YE174; (8) *S. bailii* YE191; (9) *S. cerevisiae* YE193; (10) *S. cerevisiae* YE244. Values are means of three different cultures. Bars represent SD.

After 5 h at 30°C, the mating-type (a or $\alpha$) of haploids was checked by light microscopy.

Mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) was performed on haploids, *S. cerevisiae* YE244-28(a) with high biomass and *S. kluyveri* YE39-16($\alpha$) with high ergosterol content. Auxotrophic mutants were selected on YNB and YNB containing amino acids. Auxotrophic mutants with high biomass or high ergosterol content were chosen to hybridize.

Hybridization was conducted by mass mating. Auxotrophic mutants *S. cerevisiae* YE244-28-35(a,leu) with higher biomass and *S. kluyveri* YE39-16($\alpha$,trp) with higher ergosterol content were mixed in a patch on a YEPD plate and incubated for 24 h at 30°C. The mixed culture was suspended in 2 ml of sterile water and starved for 4 h at room temperature. The suspension of starved cells was spread on a YNB plate and incubated for 2–3 days at 28°C. Colonies growing on the YNB plate were possible hybrids, for the auxotrophic parental strains were unable to grow on YNB. Identification of hybrids was conducted by testing growth on YNB, YNB with tryptophan and YNB with leucine, and by testing sporulation and mating-type [10,15].

### *Determination of the biomass and dry weight of cells*

The fermentation broth was centrifuged for 10 min at 4000 rpm. Cells were washed once with distilled water and centrifuged as above. The biomass was determined as the grams of cell wet weight per liter of culture (g/L). For determination of dry cell weight: 1 g of wet cells was weighed and air-dried for 6 to 8 h at 80°C to constant weight; 1 g of wet cells equaled about 0.2 g of dry cells.

### *Determination of the ergosterol content*

Alcoholic potassium hydroxide solution (50% KOH: $C_2H_5OH$=2:3) (10 ml) was added to 200 mg of dry cells in a 100-ml culture flask. The sample was saponified in a water bath for 3 h at 85 to 90°C. After cooling the flask to room temperature, 10 ml of *n*-heptane was added to the saponified solution and the flask was shaken vigorously about 30 times. The mixture was held at room temperature for 30 min to let the *n*-heptane layer clarify. Then, 0.5 ml of the supernatant fluid was diluted with 4.5 ml of absolute ethanol.

Absorbance was read at 281.5 and 230 nm against a blank of 0.5 ml *n*-heptane and 4.5 ml of absolute ethanol. The ergosterol content was determined as the milligram ergosterol per gram dry cells and calculated using the formula [3]:

$$\text{ergosterol content (mg/g dry cells)} = \left(\frac{\text{OD}_{281.5\,\text{nm}}}{290} - \frac{\text{OD}_{230\,\text{nm}}}{518}\right) \times \text{F}$$

where 290 is the $E^{(1\%,\ 1\ \text{cm})}$ value of crystalline ergosterol; 518 is the $E^{(1\%,\ 1\ \text{cm})}$ value of 24(28)-dehydroergosterol. *F* is the factor for sample size and dilutions.

The yield of ergosterol was determined by both biomass (or cell dry weight) and ergosterol content. It was calculated using the formula:

$$\begin{aligned}\text{ergosterol yield (mg/L)} &= \text{biomass (g/L)} \times 0.2 \\ &\times \text{ergosterol content (mg/g dry cells) or} \\ &= \text{cell dry weight (g/L)} \\ &\times \text{ergosterol content (mg/g dry cells)}\end{aligned}$$

where 0.2 means that one gram wet cells is equal to about 0.2 g dry cells.

**Table 1** Comparison of genetic characteristics and yields among hybrids and parental strains

| Strain | Ploidy | Mating type | Sporulation | Auxotroph marker | Biomass (g/L) | Ergosterol content (mg/g) | Yield of ergosterol (mg/L) |
|---|---|---|---|---|---|---|---|
| YE39 | 2*n* | a/$\alpha$ | + | | 8.5±0.7 | 73.1±1.4 | 124.3 |
| YE244 | 2*n* | a/$\alpha$ | + | | 68.6±0.5 | 10.9±0.5 | 149.5 |
| YE39-16 | *n* | $\alpha$ | − | | 10.8±0.4 | 33.9±0.7 | 73.2 |
| YE244-28 | *n* | a | − | | 44.9±0.9 | 10.1±0.2 | 90.7 |
| YE39-16-21 | *n* | $\alpha$ | − | trp | 11.2±0.3 | 38.1±0.9 | 85.3 |
| YE244-28-35 | *n* | a | − | leu | 46.4 | 11.7±0.7 | 108.6 |
| YEH-28 | 2*n* | a/$\alpha$ | + | | 27.6±0.3 | 43.6±0.4 | 240.7 |
| YEH-56 | 2*n* | a/$\alpha$ | + | | 28.4±0.5 | 43.4±0.3 | 246.5 |

Fermentation was conducted in YEPD medium for 24 h at 28°C with agitation (200 rpm). Values are means of three different cultures.
±Values represent SD.

### *Analysis of the hybrids' genetic stability*

The hybrid strains YEH-28 and YEH-56 from a YEPD slants were cultivated in 5 ml of YEPD liquid media for 14 h at 28°C on a rotary shaker. Cultures were diluted and spread on a YEPD plate. After 48 h at 28°C, 100 colonies of each hybrid strain were transferred to 1 ml of sterile $H_2O$ and starved 4 h at room temperature. Each starved suspension was inoculated on plates of YEPD, YNB, YNB with tryptophan and YNB with leucine and incubated for 48 h at 28°C. Sporulation, mating-type, biomass and cell ergosterol content of these colonies were also determined.

### *Statistical analysis*

All fermentation cultures were run in triplicate in 250-ml Erlenmeyer flasks. All data were analyzed statistically using Data Analysis and Technical Graphics, origin 5.0 (Microcal Software Inc.).

## Results

### *Preliminary screening*

The biomass and ergosterol content of 230 yeast strains from different genera and species were tested. The results for five strains having high ergosterol content and five strains having high biomass are summarized in Figure 1.

### *Construction of ergosterol-producing strains by hybridization*

According to the results of preliminary screening and sporulation tests, diploid strain YE244 (*S. cerevisiae*) with high biomass and diploid strain YE39 (*S. kluyveri*) with high ergosterol content were selected as parental strains. Haploids YE244-28(a) with high biomass and YE39-16($\alpha$) with high ergosterol content were selected by the method described above. The two haploid strains were mutagenized by NTG. The mutants YE244-28-35(a,leu) and YE39-16-21($\alpha$,trp) were selected as hybrid parental strains for their different auxotrophic markers and higher biomass or higher ergosterol content. Three times of hybridization yielded 127 hybrids. Two hybrid strains that gave a higher yield of ergosterol than the parental strains were selected. The results in Table 1 indicate that the hybrids YEH-28 and YEH-56 had the characters superior to their parental strains. The ergosterol yield of strain YEH-28 was about 1.94 and 1.61 times that of the parental strains YE39 and YE244, respectively; while the ergosterol yield of YEH-56 was about 1.98 and 1.65 times that of the parental strains YE39 and YE244, respectively.

The genetic stability of the hybrids was determined by the method described above. All single colonies of YEH-28 and YEH-56 that were checked produced spores on the sporulation medium, grew on YNB medium, and had no distinct change in biomass and ergosterol content. However, the parental strains, YE244-28-35(a,leu) and YE39-16-21($\alpha$,trp), were unable to sporulate and grow on YNB medium (Table 2). Thus, the hybrid strains YEH-28 and YEH-56 are stable enough in genetic and haven't the separation phenomena of auxotrophic markers.

**Table 3** Effect of carbon source on the biomass and ergosterol content of YEH-56

| Carbon source | Biomass (g/L) | Ergosterol content (mg/g) | Yield of ergosterol (mg/L) |
|---|---|---|---|
| Cane sugar | 29.2±0.9 | 32.1±1.6 | 187.5 |
| Glucose | 29.3±0.3 | 40.6±0.7 | 237.9 |
| Sucrose | 29.2 | 31.8±0.9 | 185.7 |

The concentration of carbon source was 2% (w/v).
Values are means of three different cultures.
±Values represent SD.

### *Factors affecting the biomass and ergosterol content in hybrid strain YEH-56*

*Effect of carbon source:* YEPD medium chosen contained 2% carbon source, 2% peptone and 1% yeast extract. The carbon sources were cane-sugar, sucrose or glucose. After 24 h fermentation, the biomass and ergosterol content of hybrid strain YEH-56 were determined. The result proved that different carbon sources had no effect on the biomass, but glucose was superior for ergosterol production (Table 3). The effect of glucose concentration on the biomass and ergosterol content was determined. The biomass was the highest at 8% glucose while the highest ergosterol content was at 16% glucose (Figure 2). Making a general survey of the biomass and ergosterol content, 10% glucose is optimal for the highest yield of ergosterol.

*Effect of nitrogen source:* Different concentrations of $(NH_4)_2SO_4$, $NH_4Cl$ or urea were added to YEPD medium with 10% glucose as carbon source. Different nitrogen sources had a

**Table 2** Genetic stability of hybrid and parental strains

| Strain | Numbers of colonies on different media | | | | Sporulation | Biomass (g/L) | Ergosterol content (mg/g) | Yield of ergosterol (mg/L) |
|---|---|---|---|---|---|---|---|---|
| | YEPD | YNB | YNB+trp | YNB+leu | | | | |
| YE39-16-21 | 100 | 0 | 100 | 0 | − | 11.4±0.5 | 38.2±0.4 | 87.1 |
| YE244-28-35 | 100 | 0 | 0 | 100 | − | 46.3±0.7 | 11.8±0.2 | 109.3 |
| YEH-28 | 100 | 100 | 100 | 100 | + | 27.9±0.5 | 43.4±0.2 | 242.7 |
| YEH-56 | 100 | 100 | 100 | 100 | + | 28.5±0.4 | 43.2±0.3 | 246.2 |

Fermentation was conducted in YEPD medium for 24 h at 28°C with agitation (200 rpm). Values are means of three cultures. ±Values represent SD.

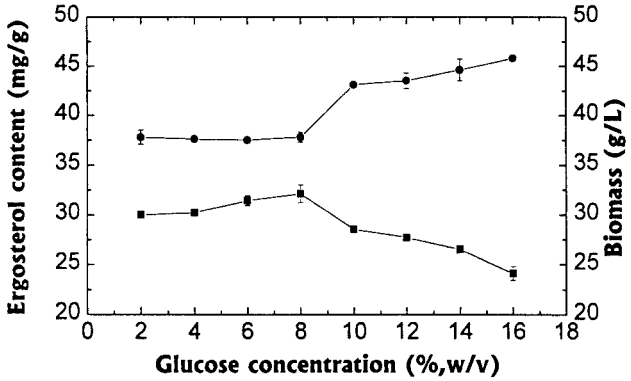


**Figure 2** Effect of glucose concentration on the biomass and ergosterol content of hybrid strain YEH56. Ergosterol content(●), biomass(■). Values are means of three different cultures. Bars represent SD.

great effect on yields (Table 4). The biomass and ergosterol content decreased, as the concentration of nitrogen source increased, especially with urea. Hence, no extra nitrogen source was added to the fermentation media for strain YEH-56 in subsequent studies.

*Size of inoculum:* The effect of inoculum volume on the biomass and the yield of ergosterol of hybrid strain YEH-56 was tested on the basis of the previous results. Inoculum volume had little influence on the biomass in the initial 24 h. Thereafter, ergosterol content was highest at 10% (v/v) inoculum volume and decreased a little with increased inoculum volume (Figure 3A). Therefore, the optimal inoculum volume for ergosterol production of hybrid strain YEH-56 was 10%.

*Effect of initial pH:* The fermentation medium was adjusted to pH levels ranging from 4.0 to 8.0. Maximum ergosterol content of the hybrid strain YEH-56 was observed at initial pH 6.0. Although biomass reached maximum at pH 6.5, considering ergosterol yield, the optimal initial pH of the fermentation medium was 6.0 (Figure 3B).

*Effect of the volume of fermentation medium:* A different volume of fermentation medium was added to each 250-ml flask. At a constant shaker speed, biomass gradually decreased with increase culture volume. When the volume was 60 ml, the ergosterol yield was the highest (Figure 3C).

*Fermentation time:* Under the above optimal fermentation conditions, the biomass and ergosterol content of strain YEH-56 were determined at different fermentation times. So during cultivation, biomass varied with the ergosterol content in 30 h. The highest biomass appeared at 55 h, but ergosterol content reached maximum at 30 h (Figure 3D). For best utilization of the equipment, the fermentation time recommended was 30 h.

*Comparison of the biomass and ergosterol content produced by hybrid and parental strains under optimal conditions:* Under optimal conditions, the biomass and ergosterol content of parental strains and hybrid strains YEH-28 and YEH-56 were compared (Figure 4). The yield of ergosterol of hybrid strains YEH-28 was 1.96 and 1.56 times that of the parental strains YE39 and YE244, respectively; while the yield of ergosterol of YEH-56 was 1.98 and 1.57 times that of the parental strains YE39 and YE244, respectively.

## Discussion

The yield of ergosterol depends on not only ergosterol content, but also biomass. High biomass was accompanied by low cell ergosterol content (or high cell ergosterol content was accompanied by low biomass) in wild strains. For example, the biomass of strain YE244 reached 68.6 g/L, but its ergosterol content was only 10.9 mg/g. The biomass of strain YE39 was only 8.5 g/L, but its ergosterol content reached 73.1 mg/g (Figure 1). This has also been observed by others [2,3].

It was difficult to get a strain with both higher biomass and higher ergosterol content by natural screening or mutagenesis [13]. However, it was possible to obtain such strains by genetic breeding technology, such as protoplast fusion [14,15] and hybridization. In this study, the principal reasons that YE244-28-35(a,leu) and YE39-16-21($\alpha$,trp) were chosen as hybridization parent strains are as follows. First, YE244-28-35(a,leu) and YE39-16-21($\alpha$,trp) have different mating-types and can mate each other. Secondly, since the auxotroph of YE244-28-35(a,leu) and YE39-16-21($\alpha$,trp) are complementary, the

**Table 4** Effect of the concentration of nitrogen sources on the biomass and ergosterol content of strain YEH-56++

| Concentration of nitrogen source (%, w/v) | Ammonium sulfate | | Ammonium chloride | | Urea | |
|---|---|---|---|---|---|---|
| | Biomass (g/L) | Ergosterol content (mg/g) | Biomass (g/L) | Ergosterol content (mg/g) | Biomass (g/L) | Ergosterol content (mg/g) |
| 0 | 29.2±0.5 | 42.2±1.7 | 29.2±0.5 | 42.2±1.7 | 29.2±0.5 | 42.2±1.7 |
| 0.2 | 29.1±0.3 | 39.3±1.3 | 28.6±0.3 | 38.6 | 28.1±0.1 | 32.1±0.7 |
| 0.4 | 26.8±0.9 | 33±0.7 | 25.5±0.5 | 30.2±1.2 | 21.1±0.3 | 18.6±0.3 |
| 0.6 | 25.4±0.4 | 30.2±0.3 | 22.4±0.1 | 28.1±0.2 | 17.8±0.2 | 15.3±0.2 |
| 0.8 | 22.9 | 25.5±0.2 | 20.5±0.2 | 25.9±0.3 | 14.4 | 13.8±0.1 |
| 1.0 | 18.4±0.2± | 22.4±0.9 | 17.7±0.7 | 23.1±0.5 | 12±0.1 | 12.8±0.7 |

Fermentation was conducted in modified YEPD medium with different concentrations of nitrogen sources for 24 h at 28°C with agitation (200 rpm). Values are means of three different cultures. ±Values represent SD.

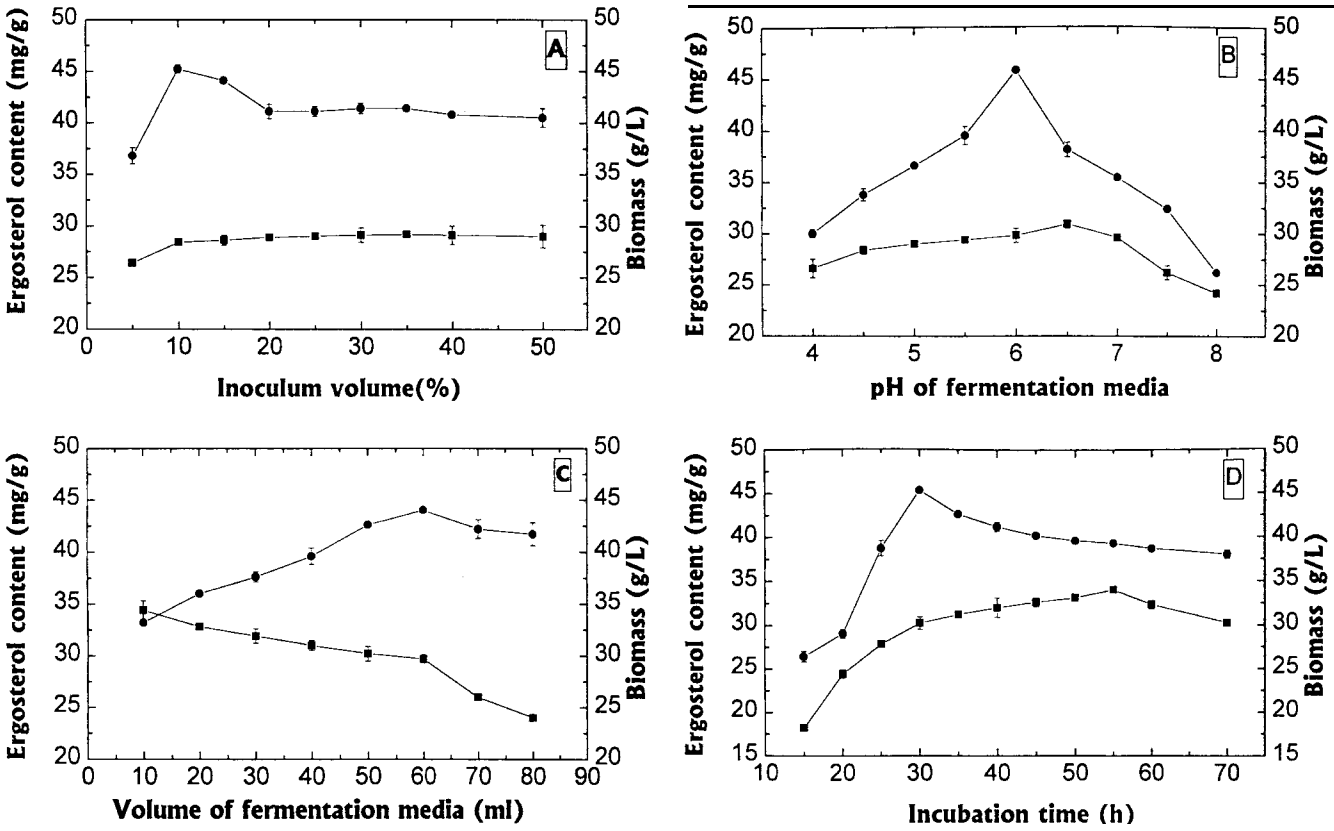


**Figure 3** Effect of the fermentation conditions on the biomass and ergosterol content of hybrid strain YEH56. Ergosterol content (●), biomass (■). Values are means of three different cultures. Bars represent SD.

hybrids can be obtained easily and selected by testing growth on YNB medium. Thirdly, YE244-28-35(a,leu) achieves higher biomass, while YE39-16-21($\alpha$,trp) possesses a higher ergosterol content. The goal of this study was to obtain strains with both higher biomass and higher ergosterol content. Hybrid strains YEH-28 and YEH-56 constructed by hybridization gave higher yields of ergosterol than their parental diploid strains YE39 and YE244. These results suggested that the superior properties of parental strains were combined.

Among the three kinds of carbon source tested, glucose was superior for ergosterol production, similar to the results of Novotny and Starr *et al.* [8] and Starr and Parks [11]. The ergosterol biosynthesis pathway can be divided into four steps: formation of mevalonic acid from acetyl-CoA, transformation of mevalonic acid into squalene, formation of lanosterol from squalene and synthesis of ergosterol from lanosterol [9,12]. The first step is important in controlling ergosterol formation. One reason for the effect of glucose on ergosterol content may be increased acetyl-CoA formation from glucose, which promotes synthesis of ergosterol. However, a high glucose concentration restrained cell growth.

The effect of nitrogen sources on the biomass and ergosterol content varied with different strains. In this study, all three nitrogen sources ammonium sulfate, ammonium chloride and urea, did not promote cell growth by biosynthesis of ergosterol in hybrid strain YEH-56.

Biosynthesis of ergosterol is a characteristic of aerobic metabolism [3]. However, poor aeration inhibited growth and stimulated synthesis of ergosterol in strain YEH-56. The reasons for this are not clear.

Analysis of genetic stability showed that all single colonies of the hybrid strains YEH-28 and YEH-56 checked produced spores on the sporulation medium, grew on YNB medium, and had no distinct change in biomass or ergosterol content. However, the parental strains for hybridization, YE244-28-35(a,leu) and

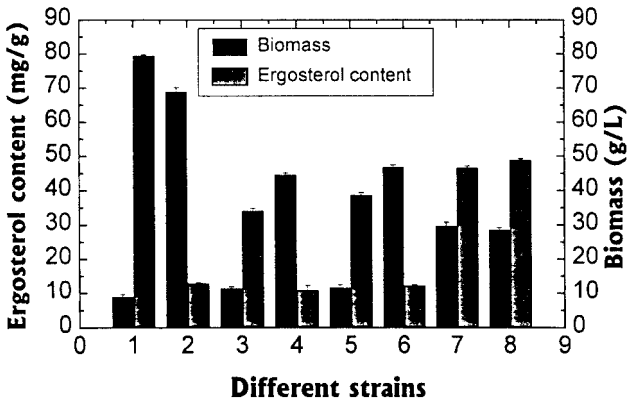


**Figure 4** Comparison of the biomass and ergosterol content among parental strains and hybrid strains under optimal conditions. (1) YE39; (2) YE244; (3) YE39-16; (4) YE244-28; (5) YE39-16-21; (6) YE244-28-35; (7) YEH-28; (8) YEH-56. Values are means of three different cultures. Error bars represent SD.

YE39-16-21(α,trp), were unable to sporulate and grow on YNB medium (Table 2). These results show that the hybrid strains YEH-28 and YEH-56 are stable in genetic and haven't the separation phenomena of auxotrophic markers.

In conclusion, hybridization is a valuable tool for improving industrial yeasts with dominant characteristics such as increasing ergosterol yield. As shown in Figure 4, hybrid strains YEH-28 and YEH-56 have a higher yield of ergosterol than their parent strains YE39 and YE244. Under optimal conditions, the yield of ergosterol of YEH-28 is 1.96 and 1.56 times that of the parent strains YE39 and YE244, while the ergosterol yield of YEH-56 is 1.98 and 1.57 times that of the parent strains YE39 and YE244, respectively. They have high genetic stability. Therefore, they have the potential for use in industrial processes.

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