微生物学报 *Acta Microbiologica Sinica*
55(1):22-32; 4 January 2015
ISSN 0001-6209; CN 11-1995/Q
http://journals.im.ac.cn/actamicrocn
doi: 10.13343/j.cnki.wsxb.20140159

Induction, separation and identification of haploid strains from industrial brewer's yeast

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Abstract: [**Objective**] Lager brewing yeasts (*Saccharomyces pastorianus*), the natural hybrids of *S. cerevisiae* and *S. eubayanus*, are usually heterothallic polyploidy or aneuploidy. Their intricate ploidy is a great challenge to genetic studies and strain improvement. Haploid breeding is an effective method to overcome these difficulties. Also, haploid strains play an important role in scientific research and breeding. However, lager brewing yeasts only divide asexually and hardly bear spores under normal conditions, so it is very difficult to get haploid strains from them. In this study, we established comprehensive methods to induce, separate and identify haploid strains of industrial brewer's yeast. [**Methods**] First, we selected efficient sporulation medium to induce the sporulation of an industrial brewer's yeast strain G-03, and then isolated spores from vegetative cells and formed colonies on YPD plates. After that, flow cytometry was used to determine the ploidy types of the pre-judged haploid candidates. Ultimately, we analyzed the genotypes of the segregants by PCR reaction and mating test in order to get precise results. [**Results**] Using this protocol, we obtained 26 yeast segregants by spore isolation, and 4 of them pre-judged as haploid candidates were finally confirmed as haploid by flow cytometric analysis. Two of them were *MAT*a and others were *MAT*α. By scanning electron microscope (SEM), the cells of 4 haploid segregants showed similar morphology to each other but had obvious differences compared with the parent strain. Pseudohyphal growth occurred in parent cells after long-period cultivation but none was found in haploid segregants. [**Conclusion**] Sporulation of industrial brewer's yeast and germination of their spores was difficult but not impossible. Nevertheless, the screening and identification of haploid segregants were more challenging.

Keywords: brewer's yeast, sporulation, haploid breeding, flow cytometry, cell morphology
CLC number: Q935 Article ID: 0001-6209(2015)01-0022-11

During beer fermentation, yeast produces numerous beer flavor compounds including esters, acetaldehyde and higher alcohols. At the same time, some other characteristics of brewer's yeast, such as

Supported by the Fundamental Research Funds for the Central Universities (JUDCF13008, JUSRP51402A), by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), by the Program for New Century Excellent Talents in University of China (NCET-10-0453), by the National Programs for High Technology Research and Development of China (2012AA021303, 2013AA102106-03) and by the National Natural Science Foundation of China (31301539, 31271919)

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Received: 29 March 2014/ Revised: 28 May 2014

cell age, fermentation rate, and flocculation, are also important in brewing industry [1-2]. So brewer's yeast has a decisive influence on beer fermentation. Brewer's yeast is divided into two groups, traditionally defined as top-fermenting yeast and bottom-fermenting yeast. Top-fermenting yeast is closely related to *S. cerevisiae* and used to produce ale. Bottom-fermenting yeast is also called lager brewing yeast (*S. pastorianus*), which is used to produce lager and contributes to 90% of the total beer market [3-4]. Hence most of the researches on brewer's yeast are conducted on lager strains.

Ploidy is a fundamental genetic characteristic with major genetic and genomic implications [5]. Lager brewing strains are generally hybrids of *S. cerevisiae* and *S. eubayanus* [6]. Diploidy, polyploidy, and aneuploidy are popular in lager strains, among which the latter two are more common. An organism carrying three or more sets of nuclear chromosomes is determined as being polyploidy, while a non-integer ploidy number of chromosomes is characterized to be aneuploidy [7]. Aneuploidy is well tolerated and stable. In previous studies, a lot of difficulties have been encountered in gene modifications and strain optimizations on brewer's yeast because of their complicated karyotypes [2,8]. However, haploid strains have only one copy of nuclear chromosomes, offering convenience in yeast metabolic and genetic studies [9-10]. Moreover, they play an important role in breeding works. Mating-competent spores can be isolated from brewer's yeast to build new yeast strains with optimized features by mating [11-12]. Although haploid strains are indispensable in research, there is little information about the systematic methods for breeding the haploid of brewer's yeast. The most likely reason is that brewer's yeast is currently thought to have very poor sporulation ability and low degree of spore viability [11].

Flow cytometry (FCM) is a technique which could quantitatively analyze and sort biological particles or cells present in a suspension. It studies microorganism populations on individual cell level, different from traditional biochemical methods which studies microorganisms in bulk. Thus the veracity and specificity are well guaranteed. FCM method is also time-saving because of the high velocity [13-14]. It is now recognized as an essential tool for quantitative assays of microorganisms [15-16]. In recent years, FCM has been adopted to analyze the genome size and ploidy of fungi, *Zygosaccharomyces* species [17], sake yeast [18], *Schizosaccharomyces pombe* [19], for instance. However, few attempts have been made to study brewer's yeasts' ploidy by FCM so far.

In this study, special attentions were drawn to establish systematic methods for haploid breeding from industrial brewer's yeast. There were five procedures in our scheme: sporulation, spore isolation, pre-judgment, final-judgment, and genotype detection. The yeast G-03 was used for haploid breeding and ultimately 4 haploid segregants were obtained, among which 2 were determined as *MAT*a and 2 were *MAT*α. In addition, the cell morphology of 4 haploid segregants and their parent strain G-03 were examined by SEM.

1 Materials and Methods

1.1 Strains and culture conditions

Yeast strains used in this study were listed in Table 1. G-03, an industrial heterothallic brewer's yeast, was applied to culture haploid segregants. W303-1A (haploid, *MAT*a) and W303-1B (haploid, *MAT*α) were used as reference standards for ploidy determination and *MAT*-PCR, also as tester strains for mating test. Yeast strains were routinely cultured in YPD medium (20 g/L peptone, 20 g/L glucose, and 10 g/L yeast extract). 20 g/L agar was added for solid medium.

Table 1. Yeast strains used in this study

Strain	Genotype	Source or reference
G-03	Industrial strain for beer brewing, *MAT*a/α	Fengtai beer brewery (Anhui province, China)
W303-1A	*MAT*a leu2-3/112 his3-11/15 trp1-1 ura3-1 ade2-1 can1-100	[20]
W303-1B	*MAT*α leu2-3/112 his3-11/15 trp1-1 ura3-1 ade2-1 can1-100	[20]
H 2	Haploid segregant of G-03, *MAT*α	This study
H 5	Haploid segregant of G-03, *MAT*α	This study
H12	Haploid segregant of G-03, *MAT*a	This study
H18	Haploid segregant of G-03, *MAT*a	This study

1.2 The selecting of the optimal sporulation medium

Considering the poor sporulation ability of brewer's yeast, 5 media were prepared. They were Kleyn medium (2.5 g/L peptone, 0.62 g/L glucose, 0.62 g/L NaCl, and 5 g/L NaAc), McClary medium (1 g/L glucose, 2.5 g/L yeast extract, 1.8 g/L KCl, and 8.2 g/L NaAc), modified McClary medium (McClary medium with 0.125 g/L $ZnSO_4 \cdot 7H_2O$, 2 μg/L biotin, and 100 μg/L inositol), potassium acetate sporulation medium (SPM; 1 g/L glucose, 2.5 g/L yeast extract, 10 g/L KAc, and 1 g/L KH_2PO_4) and modified SPM medium (SPM medium with 23.6 g/L succinic acid, 0.125 g/L $ZnSO_4 \cdot 7H_2O$, 2 μg/L biotin, and 100 μg/L inositol, pH 6.0), respectively. G-03 was firstly activated in YPD medium. Then the yeast suspensions were diluted and transferred to sporulation plates, and incubated at 28℃ for 7 d. After that, cells were stained by malachite green-safranine staining method [11]. The ascospores were found to be stained in green, and vegetative cells were in red when observed by microscope. In order to select the best sporulation medium, approximately 1000 to 1500 cells were counted to calculate the sporulation rate according to the Eq. 1. For the budding yeast cells, if the daughter cell was larger than half size of the mother cell, it was counted as one cell.

Eq. 1 Sporulation rate = Number of spores / Total number of cells × 100%

1.3 Spore isolation and enrichment

Cells which had formed spores were suspended in sterilized saline (0.9%, W/V) and harvested by centrifugation (2000 ×g for 5 min). Then 20 mg/mL snailase (BBI, Konrad Cres, Markham Ontario, Canada) was added to the suspension. The mixture was placed in a 37℃ water-bath for 2 h, and then transferred to a 58℃ water-bath for 10 min to kill the vegetative cells. Afterwards, the cells were spread on YPD plates and cultured at 28℃ for 3 d to allow colony formation.

1.4 Pre-judgment of haploid state

Random chosen colonies were transferred to sporulation plates. During 3 weeks' cultivation, cells were regularly stained using the malachite green-safranine staining method and processed for microscopic examination. Cells that did not form spores invariably could be pre-judged as haploid segregants and used for further studies. Others were used to conduct spore isolation again.

1.5 Determination of ploidy by flow cytometry

The ploidy types of pre-judged haploid segregants were determined by flow cytometric analysis. These segregants were cultured in YPD medium at 28℃ overnight and harvested by centrifugation. After resuspended and diluted in phosphate-buffered saline (PBS; 8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4, pH 7.4), the cells were placed in a 100℃ water-bath for 10 min and then treated with ultrasound for 20 – 30 s. 5 μL 1 mg/mL propidium iodide (PI; Sigma, St. Louis, MO, USA) was added to 1 mL suspension and the mixture were kept in dark for 5 min. Control samples were prepared in the same way. The ploidy of the samples was determined by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ,

USA). Ultrapure water was used as sheath fluid. A 15 mW laser with a wavelength of 488 nm was used to excite the fluorescence of PI and the fluorescence was measured by a 660/16 nm band pass filter. The flow rate was adjusted to keep the rate below 1000 events per second, and a minimum of 40,000 cells were acquired for each sample. DNA ploidy was automatically determined from DNA histograms, which were generated by the CellQuest Pro software (BD Biosciences).

1.6 Analysis of the *MAT* locus by PCR

PCR was implemented directly on colonies of yeast according to Huxley's statement [21] and modified. Ex Taq was chosen as DNA polymerase (TaKaRa Bio, Dalian, China). The PCR reaction was carried out at 94℃ for 5 min, followed by 30 cycles of 94℃ for 1 min, 50℃ for 30 s and 72℃ for 1 min, finally extended at 72℃ for 5 min. The PCR products were electrophoresed on 2% agarose gels. For laboratory strains of yeast, haploid colonies yield either the *MAT*a-specifific product of 544 bp or *MAT*α-specific product of 404 bp corresponding to their mating type, while diploid colonies yield both products (homozygous diploid or polyploid yield only one product).

1.7 Mating test

The mating test was carried out according to the background and principle of yeast mating reaction described by Sprague GF [22]. The mating results were examined by microscopic observation and flow cytometric analysis. If the haploid segregant could mate with W303-1A (haploid, *MAT*a), it was preliminarily judged as *MAT*α. If it could mate with W303-1B (haploid, *MAT*α), it was estimated as *MAT*a. Haploid segregants unable to mate with either haploid tester were considered to be sterile.

1.8 Analysis of yeast cell morphology

G-03 as well as haploid segregants were cultured on YPD for 24 h and 60 h. Then the cell morphology was examined by scanning electron microscope (SEM, HITACHI, Tokyo, Japan) at a 10.0 kV acceleration voltage and the image magnification was 8000.

2 Results

2.1 Determination of the optimal sporulation medium

The flowchart of haploid breeding was shown in Figure 1. When sporulation media were selected, cells were stained by malachite green-safranine staining method followed by microscopic examination to calculate the sporulation rate. It was found that most asci contained 2 - 3 spores, 4-spore ones were scarce. The sporulation rates of G-03 on Kleyn, McClary, modified McClary, SPM and modified SPM medium were shown in Figure 2. Yeast strain can hardly bear spores on Kleyn medium. The highest sporulation rate emerged on modified McClary medium (29.6% ± 1.8%), higher than that on McClary medium (25.6% ± 1.6%). The sporulation rate of G-03 on modified SPM medium (24.1% ± 1.4%) was also higher than that on SPM medium (6.5% ± 1.3%). Modified McClary medium had the highest sporulation rate, thus was selected as the sporulation medium for further research.

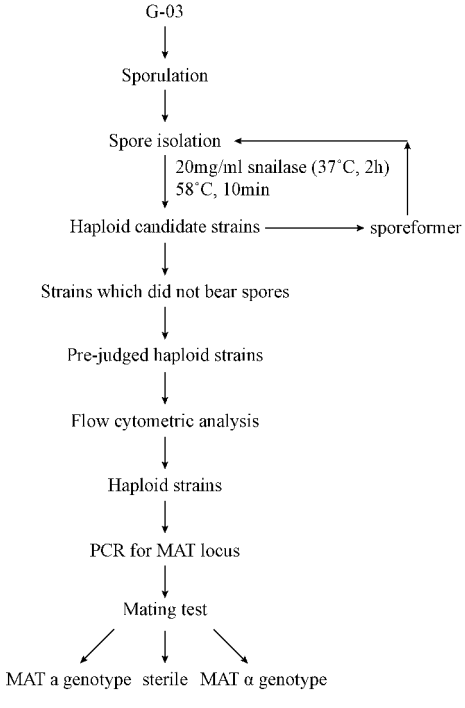

Figure 1. The flowchart of haploid breeding.

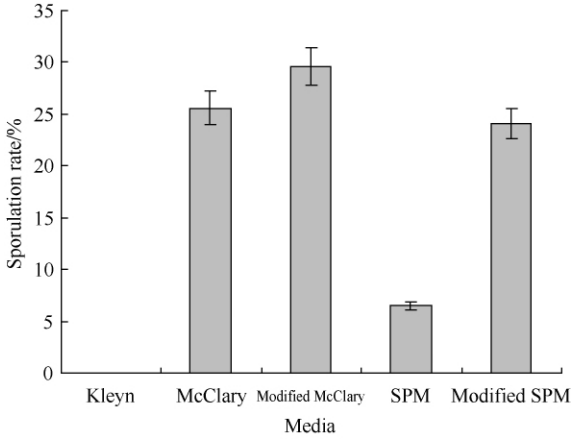

Figure 2. The sporulation rates of G-03 on Kleyn, McClary, modified McClary, SPM, and modified SPM medium. The rates were respectively 0%, 25.6% ± 1.6%, 29.6% ± 1.8%, 6.5% ± 1.3% and 24.1% ± 1.4%.

2.2 Separation and identification of haploid segregants

After spore isolation, 26 segregants derived from G-03 were obtained and all these colonies were transferred to modified McClary plates to examine whether they could bear spores again. One segregant bore spores in a week, 21 segregants bore spores in the following two weeks, and 4 segregants did not bear spores all the way during 3 weeks' cultivation. Thus, the 4 segregants were chosen as haploid candidates (named H2, H5, H12, and H18) for flow cytometric analysis. In order to get precise results, *S. cerevisiae* W303-1B were used as standard haploid strains. The results of FCM DNA analysis on W303-1B, G-03 and 4 haploid candidates were shown in Figure 3A, 3B, 3C, and Table 2. Peak position and mean fluorescence intensity in DNA histograms were two decisive parameters which reflected the ploidy of strains. The fluorescence intensity is proportional to the DNA content. Peak positions of the 4 haploid candidates (117 ± 0, 117 ± 0, 119.67 ± 4.62, and 117 ± 0) were closely to that of W303-1B (122.33 ± 4.62).

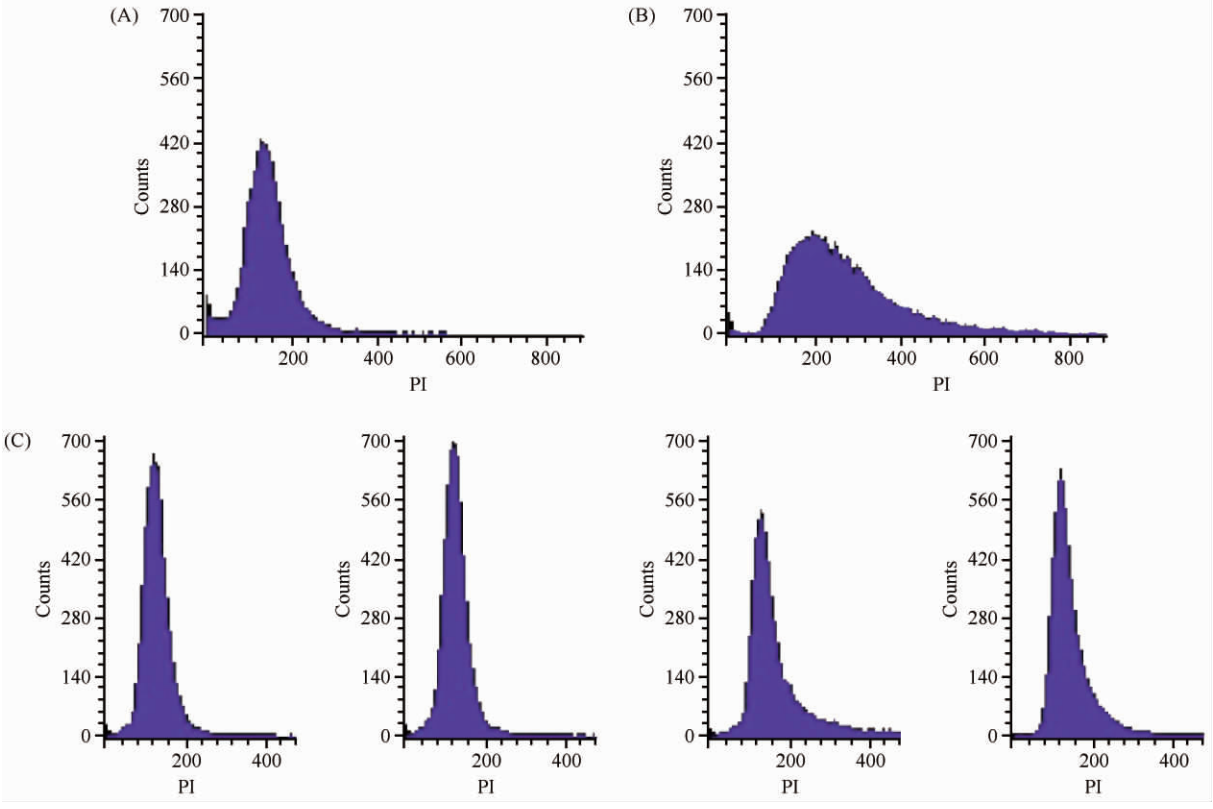

Figure 3. Identification of haploid segregants. (A) FCM profile of W303-1B; (B) FCM profile of G-03; (C) FCM profiles of 4 haploid segregants H2, H5, H12, and H18. Peak positions of the 4 haploid candidates were respectively 117 ± 0, 117 ± 0, 119.67 ± 4.62, and 117 ± 0, which were all close to that of W303-1B (122.33 ± 4.62). Moreover, their mean DNA contents were also approximate to standard haploid strain. Thus, these 4 segregants were determined as haploid strains.

Moreover, their mean DNA contents (135.52 ± 1.71, 134.15 ± 1.57, 141.99 ± 1.08, and 142.17 ± 0.59) were also approximate to the standard haploid strain (141.50 ± 0.57). Thus, these 4 segregants were determined as haploid strains. In addition, the peak shapes of 4 haploid segregants and W303-1B were similar, which were all narrow and pointed, indicating a high degree of purity. On the contrary, G-03 had a wide and flat peak, reflecting a high variation coefficient.

Table 2. Results of FCM DNA analysis and mating test

Strains	Peak position of fluorescence intensity	Mean fluorescence intensity	Genotype
W303-1B	122.33 ± 4.62	141.50 ± 0.57	*MAT*α
G-03	200.67 ± 4.04	277.37 ± 1.63	*MAT*a/α
H2	117 ± 0	135.52 ± 1.71	*MAT*α
H5	117 ± 0	134.15 ± 1.57	*MAT*α
H12	119.67 ± 4.62	141.99 ± 1.08	*MAT*a
H18	117 ± 0	142.17 ± 0.59	*MAT*a

The mating type of the 4 segregants was examined by colony *MAT*-PCR. H2 and H5 yielded a product of 404 bp, indicating that they were *MAT*α. H12 and H18 yielded a product of 544 bp, demonstrating the mating type of *MAT*a (Figure 4). Also, mating test was implemented to confirm their mating type and make clear whether they were sterile. After 3 hours' mating test, the cells were examined by microscopic observation every 2 h till there were enough fusion cells (about 20 – 24 h). Then the cells were implemented for FCM analysis. W303-1A (*MAT*a) and W303-1B (*MAT*α) were used as tester strains. As shown in Figure 5A and 5B, H12 could mate with W303-1B (positive) but could not mate with W303-1A (negative), so H12 was estimated as *MAT*a. The genotypes of the other 3 segregants were evaluated in the same way and results showed that H18 was *MAT*a, while H2 and H5 were *MAT*α (Table 2).

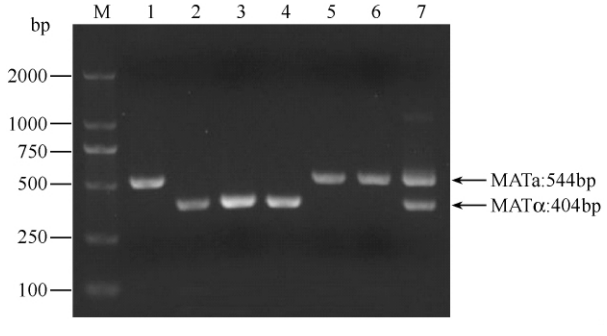

Figure 4. PCR-AE profile for *MAT* locus. Products of 544 bp and 404 bp respectively stand for *MAT*a and *MAT*α. M, DNA size marker; lane 1, W303-1A (*MAT*a), 544bp; lane 2, W303-1B (*MAT*α), 404bp; lane 3, H2 (*MAT*α), 404bp; lane 4, H5 (*MAT*α), 404bp; lane 5, H12 (*MAT*a), 544bp; lane 6, H18 (*MAT*a), 544bp; lane 7, G-03 (*MAT*a/α), 544bp and 404bp.

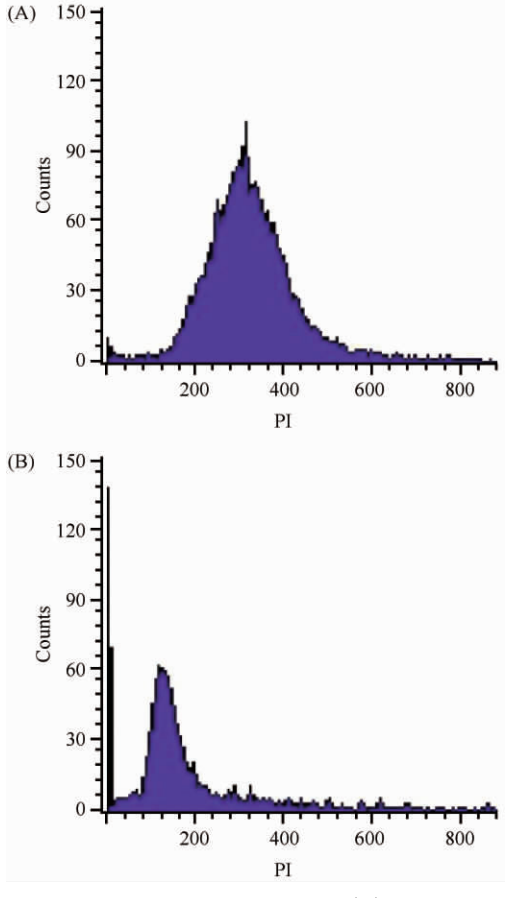

Figure 5. FCM profile of mating samples. (A) Positive result: the mating result of W303-1B and H12. The histogram showed that the sample was a diploid strain, representing that H12 could mate with W303-1B. (B) Negative result: the mating result of W303-1A and H12. It meant that H12 could not mate with W303-1A.

2.3 Cell morphology by SEM

The cell morphology of 4 haploid segregants was similar to each other but had obvious differences compared with the parent strain. The haploid cells were smaller in size, elongate, and ovoid (Figure 6B). On the contrary, G-03 had larger and rounder cells (Figure 6A). Pseudohyphal growth occurred in parent cells after long-period cultivation but none was found in haploid segregants (Figure 6C and 6D).

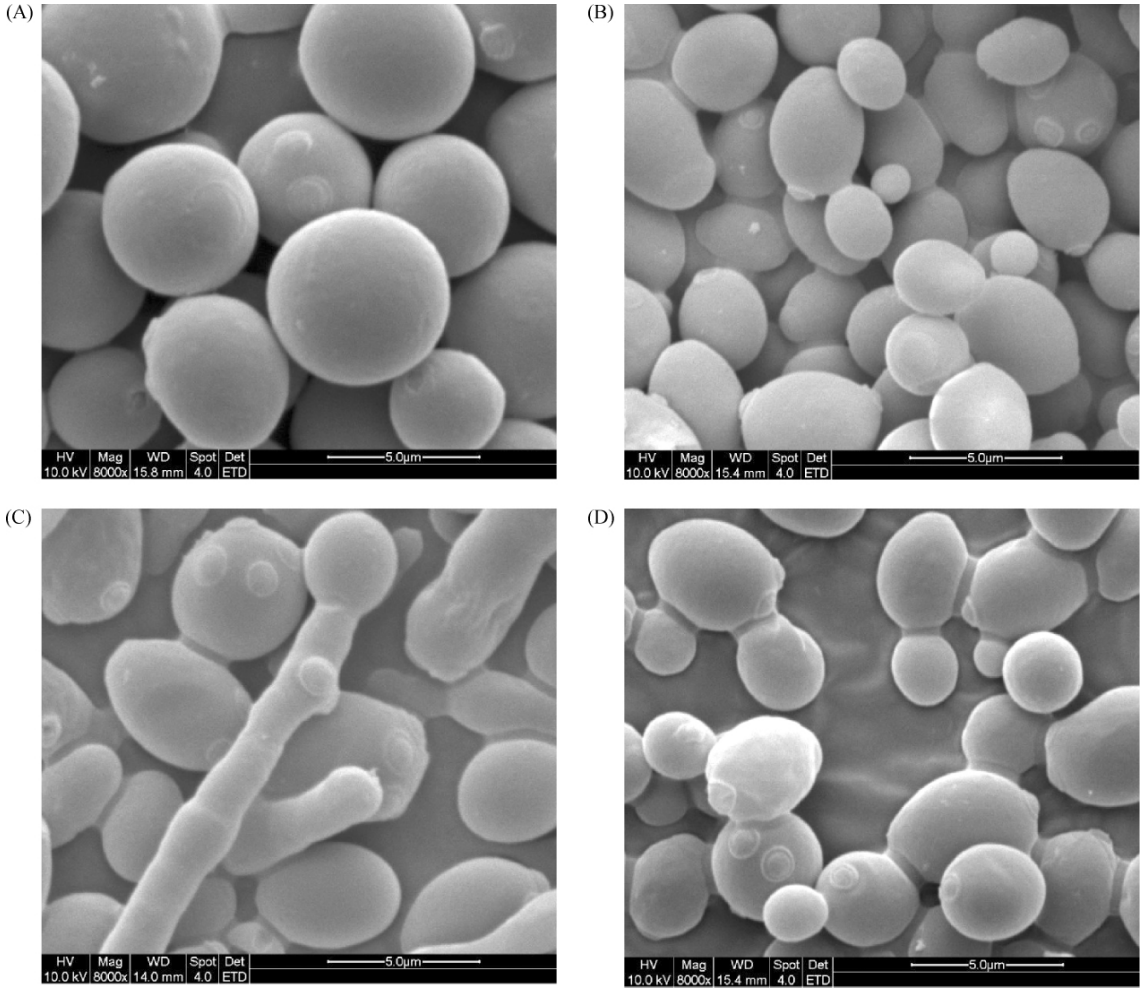

Figure 6. SEM images of yeast cells grown on YPD medium. (A) G-03 cultured for 24 h, the cells were larger and rounder; (B) H12 cultured for 24 h, the cells were smaller in size and a little oval; (C) G-03 cultured for 60 h, pseudohyphal growth occurred obviously; (D) H12 cultured for 60 h, pseudohyphal growth was not found.

3 Discussion

Haploid induction is the committed step of yeast genetic research and breeding work, because haploid strains are not only essential for characterization genetic analysis, but also serve as parental strains for crossbreeding. Moreover, they have only one copy of nuclear chromosomes and can remove troubles caused by the intricate ploidy of industrial brewer's yeast. Thus, systemic methods were established in this study to screen and identify haploid segregants induced from industrial brewer's yeast.

The sporulation was subject to regulation in accordance to the nutritional situation of the cell [23]. Moreover, most industrial brewer's yeasts were polyploidy or even aneuploidy, hence the course of second meiotic division could not be accomplished regularly and the number of spores was affected. As a result, it was found that 2 – 3 spores asci were common

and 4-spore ones were scarce when the yeast was cultured on most of the sporulation media, and the sporulation rate also varied a lot. It has previously been reported that low-level of nitrogen, carbon, and the addition of non-fermentable carbon source such as acetates could induce sporulation of yeast cells [24-25], so all the 5 sporulation media were lack of carbon source and rich in inorganic salts. In modified McClary medium, Zn^{2+}, biotin as well as inositol were added in addition of all the components in McClary medium. Besides that, succinic acid was also added to modified SPM medium. Succinic acid could adjust the pH of the medium to a stable range. Zn^{2+} could increase the activity of enzymes involved in meiosis and significantly increase the yield of 3-4 spored asci [26]. Biotin and inositol could promote the completion of the second meiotic division. As expected, the sporulation rate on modified media (modified McClary medium and modified SPM medium) was higher than that on their respective basic media (McClary medium and SPM medium). The highest sporulation rate appeared on modified McClary medium containing the wide selection of inorganic ions, such as K^+, Na^+, Cl^-, Ac^-, and Zn^{2+} besides biotin and inositol, which might be helpful in the formation of 3-4 spored asci. In fact, the 3-4 spore proportion was much higher on modified McClary medium than other media (data not shown), making a lot of sense with the haploid breeding. Accordingly, modified McClary medium was considered as the optimal sporulation medium.

Sporulation of industrial brewer's yeast and germination of their spores have been considered difficult, because industrial brewer's yeast is generally cultured under appropriate culture conditions and there is no need for the yeast to reproduce sexually. It took us several months to induce the sporulation of brewer's yeast. We also found that spore isolation was needed to repeat 1-2 times or even more in order to get real haploid segregants, because the parent strain was usually allopolyploid. Moreover, it seemed increasingly difficult for the ploidy-diminished strains to form spores. Also, the screening and identification of haploid segregants were trickier. Efficient enrichment methods made it easy to get spores. In this study, we used heat treatment, which was ordinary but useful. It was not equipment restricted and could obtain haploid segregants successfully. Although the sporulation rate was not high, spores could be readily observed with microscopic examination after stained by malachite green-safranine. The haploid segregants had special characteristics in comparison with the parent strain. They were smaller in cell size and colony size, and they grew more slowly. Pseudohyphal growth might occur in parent cells under conditions of nitrogen deficiency but none was found in haploid segregants [27]. These characteristics served as supplementary evidences to distinguish cells of different ploidy types.

DNA content is the only factor that is closely related to ploidy state under all nutrient conditions. FCM can provide further information on ploidy because the specific fluorescence intensity is proportional to the DNA amount within a single yeast cell [28]. In the *S. cerevisiae*, DNA replication initiates at many discrete origins located throughout the genome, so complete and partial duplications may occur simultaneously. Meanwhile, abnormal chromosome segregation resulting from spontaneous mutations may cause the deletion of individual chromosomes. These cases could not be excluded during sporulation although we practiced strict protocols to suppress interference in the screening of haploid strains. To confirm or eliminate these circumstances, all chromosomes should be examined by pulsed field chromosome electrophoresis or comparative genome hybridization using DNA microarray [18,29].

The genotypes of haploid segregants were assayed by colony *MAT*-PCR and mating test. The results showed that G-03 was heterozygous, because it bore spores of different mating types. Mating test was a classical method to analyze the genotype of haploid strains. Besides the mating type, it could also tell whether the strains were sterile. But only by mating test, it was not enough to confirm the genotype of yeast strains. Meanwhile, *MAT*-PCR could not remove

interferences of homozygous diploid or polyploidy. In order to get more rigorous results, *MAT*-PCR and mating test were implemented sequentially in our protocol. Furthermore, Diploid and polyploid strains with interesting brewing characteristics could be obtained by mating of haploid ones.

Ploidy reduction is often observed in *S. cerevisiae* when cultured for numerous generations, and aneuploidy also occurs. This may be a consequence of the strong natural selection imposed on yeast strains because genome plasticity provides genetic diversity [30]. So some homologous strains have similar but not the same size of chromosome. Thus they are hard to be distinguished only by FCM analysis. Meanwhile, it also implies that industrial yeast strains may not be the same ploidy as their original strain, and some may even be mixed with strains of different ploidy, but not pure culture. This may cause the high variation coefficient of industrial brewer's yeast, and may be one reason why it is difficult to make great progress in genetic research and strain improvement of industrial brewer's yeast.

Plenty genetic studies on industrial brewer's yeast are conducted on haploid strains, indicating that the haploid strains have high value in scientific research and breeding works [4]. Systemic methods for haploid breeding will be helpful in further studies. However, more researches should be done on the growth and brewing characteristics of the haploid segregants obtained in this work in order to screen for various properties to produce specific and better fermentation traits.

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啤酒酵母工业菌株单倍体的诱导、分离和鉴定

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摘要：【目的】探索适宜的方法进行啤酒酵母工业菌株单倍体的诱导、分离和鉴定，为啤酒酵母改良和遗传学研究提供便利。【方法】首先，选择产孢效果最好的培养基进行产孢诱导，诱导产生的孢子在 YPD 培养基上形成菌落后，用流式细胞技术检测其 DNA 含量，进而判断其倍性；单倍体菌株的交配型通过 *MAT*-PCR 和杂交实验确定。【结果】啤酒酵母工业菌 G-03 通过产孢诱导和孢子分离、富集后得到 26 株菌，最终通过流式细胞技术确定了其中 4 株为单倍体，*MAT*a 和 *MAT*α 型各 2 株。通过扫描电镜法观察 4 株单倍体菌株及出发菌 G-03 的细胞形态，发现单倍体菌株的形态和出发菌有较大区别，单倍体菌株长期培养没有假丝生长的现象发生。【结论】啤酒酵母工业菌单倍体育种较为困难，严格的单倍体筛选、鉴定尤其具有挑战性。

关键词：啤酒酵母，产孢，单倍体育种，流式细胞分析，细胞形态

中图分类号：Q939 **文章编号**：0001-6209 (2015) 01-0032-11

（本文责编：王晋芳）

基金项目：中央高校基本科研业务费专项资金资助（JUDCF13008，JUSRP51402A）；江苏高校优势学科建设工程资助项目（PAPD）；教育部新世纪人才支持计划项目资助（NCET-10-0453）；国家“863 计划”（2012AA021303，2013AA102106-03）；国家自然科学基金项目（31271919，31301539）

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收稿日期：2014-03-29；**修回日期**：2014-05-28