African Journal of Biotechnology Vol. 11(8), pp. 2047-2056, 26 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1581
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Effect of mating types on amorpha-4, 11-diene production in engineered *Saccharomyces cerevisiae*

Huang Yong[1,2], Kong Jian qiang[1]*, Shen Jun hao[1], Zheng Xiao dong[1], Wang Wei[1], Yang Kang juan[2] and Zhu Ping[1]*

[1]Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (State Key Laboratory of Bioactive Substance and Function of Natural Medicines & Ministry of Health Key Laboratory of Biosynthesis of Natural Products),1 Xian Nong Tan Street, Beijing 100050, China.
[2]Department of Cell Biology and Medical Genetics, College of Basic Medicine, Yanbian University, Yanji 133000, China.

Accepted 23 September, 2011

Amorpha-4,11-diene is the precursor of artemisinin, an antimalarial drug. The effect of yeast mating types on the production of amorpha-4,11-diene was investigated with the aim of improving the yield of the metabolite in engineered *Saccharomyces cerevisiae*. A yeast expression vector pYeDP60/GAPDH/ADS harbouring the amorpha-4,11-diene synthase (ADS) gene was transformed into *S. cerevisiae* W303-1A and W303-1B, which showed a and α (alpha) mating types, respectively. We also investigated the effects of four variables (carbon source, glucose concentration, nitrogen source and pH) on the fermentative production of amorpha-4,11-diene by the different mating types. Only slight differences were observed in the yields of amorpha-4,11-diene produced by the engineered yeasts, W303A[ADS] and W303B[ADS]. There were also no significant differences in the amounts of amorpha-4,11-diene produced under various growth conditions. The data generated in this study suggest that mating types of *S. cerevisiae* had no influence on amorpha-4,11-diene production levels and, therefore, either of the two mating types could be used as the parent strain of engineered yeasts.

Key words: Amorpha-4,11-diene, engineered yeasts, mating types, *Saccharomyces cerevisiae*

INTRODUCTION

Artemisinin, isolated from the plant, *Artemisia annua* L. (sweet wormwood), is the spearhead of recent anti-malarial chemotherapy (Abdin et al., 2003). Artemisinin and its semi-synthetic derivatives such as dihydro-artemisinin, artesunate and artemether are also undergoing early research and test for the treatment of cancer, especially as their mechanism of action is so different from that of other anti-cancer drugs (Chen et al., 2009; Efferth et al., 2007; Hou et al., 2008). However, artemisinin and its derivatives are not available to the millions of the world's poorest people because of the labour-intensive and time-consuming production process and inconsistent yields after a long growing season. High cost and an unreliable supply chain for artemisinin demands a new approach to production. Owing to the great progress that has been made in metabolic engineering of artemisinin (Martin et al., 2003; Ro et al., 2006; Anthony et al., 2009; Tsuruta et al., 2009; Redding-Johanson et al., 2011), the use of engineered microbes has become one of the primary concerns in an alternative production mode which could dramatically reduce the cost of artemisinin and meet global need.

Some of the engineered microbes that have been used in metabolic engineering of artemisinin include *Escherichia coli* (Martin et al., 2003), *Saccharomyces cerevisiae* (Ro et al., 2006) and *Aspergillus nidulans* (Lubertozzi et al., 2008). *S. cerevisiae* is an attractive host for the expression of plant terpenoid synthases because it can produce farnesyl pyrophosphate (FPP) for its sterol biosynthesis. Moreover, *S. cerevisiae* offers a promise for heterologous expression of the membrane-bounding protein genes that hardly have functions in bacterial systems. It has been reported that engineered yeast can produce a 2 to 3-fold level of artemisinic acid

*Corresponding author. Email: jianqiangk@imm.ac.cn. zhuping@imm.ac.cn Tel: +86(10)-63165196. Fax: +86(10)-63017757.

as compared to *in planta* production in *A. annua*, on a dry weight basis within a much shorter time; four to five days for yeast and several months for the plant (Ro et al., 2006). Consequently, *S. cerevisiae* is seen as a preferred host for microbial production of artemisinin (Ro et al., 2006).

S. cerevisiae can stably exist as either a or α (alpha) haploid and thus display simple sexual differentiation. Previous reports showed that both the a and α mating types of yeast can produce artemisinin precursors (Lindahl et al., 2006; Ro et al., 2006). Due to selection and the need to identify high-producing cell lines at an early stage in cell-line development, the effect of mating type on amorpha-4,11-diene production needs to be investigated. In this study, we constructed two engineered yeast strains; both can produce amorpha-4,11-diene but have different mating types only. We also investigated the effects of four growth variables (carbon source, glucose concentration, nitrogen source and pH) on the fermentative production of amorpha-4,11-diene by engineered yeasts with different mating types.

MATERIALS AND METHODS

Strains and plasmids

E. coli strain TG1 was used as a bacterial host for recombinant plasmid amplification. The strain was grown in LB medium (10 $g \cdot l^{-1}$ Bacto-Tryptone, 5 $g \cdot l^{-1}$ Bacto-yeast extract, 10 $g \cdot l^{-1}$NaCl) supplemented with ampicillin (100 $\mu g \cdot ml^{-1}$) when required for selection. The yeast strain *S. cerevisiae* W303-1A (*MAT*a; *ade2*-1; *his3*-11,-15; *leu2*-3, -112; *ura3*-1; *trp1*-1) and W303-1B(*MAT*α; *ade2*-1; *his3*-11,-15; *leu2*-3, -112; *ura3*-1; *trp1*-1) was stocked in our laboratory. All yeast strains were grown either in the non-selective_yeast extract-peptone-glucose (YPD) medium (10 $g \cdot l^{-1}$ yeast extract, 20 $g \cdot l^{-1}$ Bacto-peptone, 20 $g \cdot l^{-1}$ glucose) or in the selective synthetic complete (SC) drop-out medium (0.67% Bacto-yeast nitrogen base without amino acid, 2% glucose, 0.2% drop-out mixture) at 30°C.

The yeast expression vector pYeDP60/GAPDH/ADS was reported previously in which the full-length *ADS* gene was controlled by glyceraldehyde-3-phosphate dehydrogenase yeast (GAPDH) promoter (Kong et al., 2009).

Standard substance valencene was from Fluka Co. Ltd (USA). Dodecane, purchased from TCI Co. Ltd (Japan), was added to shake flask cultures (2%, v/v) and used as a layer to trap volatile amorpha-4,11-diene. All other fine chemicals were of analytical grade.

Yeast transformation

The yeast expression vector pYeDP60/GAPDH/ADS was transformed into *S. cerevisiae* W303-1A and W303-1B by the LiAc/SS carrier DNA/PEG method according to Gietz et al. (2007) individually, to construct two kinds of engineered yeasts; W303A[ADS] and W303B[ADS]. The two have the same characterization except mating types. Transformants were selected using SC-Ura-Ade drop-out medium (0.67% Bacto-yeast nitrogen base without amino acid, 2% glucose, and 0.2% drop-out mixture without uracil and adenine). Verification of positive clones was done by extraction of yeasts plasmid and further colony PCR.

Yeast cultivation and determination of amorpha-4,11-diene

All optical densities at 600 nm (OD_{600}) measurements were taken using an Agilent 6010 spectrophotometer. These engineered yeasts were grown in 10 ml SC-Ura-Ade drop-out liquid medium at 30°C to OD_{600} between 1 and 2. Then, the 1 ml culture was inoculated into 50 ml fresh YPD medium to continue cultivation three to four days at 30°C. All flasks also contained 2 ml dodecane. 200 µl dodecane layer was sampled and 1 µl out of the 200 µl dodecane was quantified for amorpha-4,11-diene production by gas chromatography-mass spectrometry (GC-MS).

GC-MS analysis of amorpha-4,11-diene

GC-MS analysis of amorpha-4,11-diene was performed as described by Lindahl et al. (2006). GC–MS conditions were as follows: SHIMADZU QP2010 system (Japan) equipped with a DB-5ms column (30 m × 0.25 µm × 0.25 mm); an injector temperature of 250°C; flow rate of 2 ml min^{-1}; split ratio of 10:1; oven temperature of 100°C for 2 min; 5°C min^{-1} that was increased to 200°C, 25°C min^{-1} increased to 250°C, and 250°C for 25 min. Total ion and selected ion chromatogram through GC–MS analysis showed molecular ion and selected ion of *m*/*z* 204 and *m*/*z* 119 and 189, respectively.

Effect of different carbon sources on the amorpha-4,11-diene production

Glycerol, xylose, sucrose, fructose (D-fructose), galactose, glucose and maltose, each at a concentration of 2% in YPD medium were used; to determine their effect on amorpha-4,11-diene production of engineered yeasts. W303A[ADS] and W303B[ADS] were grown in 10 ml SC-Ura-Ade drop-out liquid medium at 30°C to OD_{600} between 1 and 2. Then, the 1 ml culture was inoculated into 50 ml fresh YPD medium that was enriched with 2% of one of the investigated carbon sources, to continue cultivation three to four days at 30°C. All flasks also contained 2 ml dodecane. 200 µl dodecane layer was sampled and 1 µl out of the 200 µl dodecane was quantified for amorpha-4,11-diene production by GC-MS.

Effect of different glucose concentrations on the amorpha-4,11-diene production

Following the results obtained from screening of the carbon source, the effect of glucose concentrations on amorpha-4,11-diene production in engineered yeasts was studied in YPD medium with 0.4, 1, 2, 3 and 4% of glucose. W303A[ADS] and W303B[ADS] were grown in 10 ml SC-Ura-Ade drop-out liquid medium at 30°C to OD_{600} between 1 and 2. Then, 1 ml culture was inoculated into 50 ml fresh YPD medium with different glucose concentrations (0.4, 1, 2, 3 and 4%) to continue cultivation for three to four days at 30°C. All flasks also contained 2 ml dodecane. 200 µl dodecane layer was sampled and 1 µl out of the 200 µl dodecane was analyzed for amorpha-4,11-diene production by GC-MS.

Effect of different nitrogen sources on the amorpha-4,11-diene production

The nitrogen sources tested were yeast extract (Oxoid, England), urea, NH_4Cl, NH_4NO_3 and NH_4SO_4 at concentrations of 1%. W303A[ADS] and W303B[ADS] were grown in 10 ml SC-Ura-Ade drop-out liquid medium at 30°C to OD_{600} between 1 and 2. Then, the 1 ml culture was inoculated into 50 ml fresh YPD medium containing 1% different nitrogen sources to continue cultivation for

three to four days at 30°C. All flasks also contained 2 ml dodecane. 200 µl dodecane layer was sampled and 1 µl out of the 200 µl dodecane was quantified for amorpha-4,11-diene production by GC-MS.

Effect of different pH value on the amorpha-4,11-diene production

The effect of initial pH in YPD medium on the amorpha-4,11-diene production was tested. The pH of YPD medium was adjusted in one pH increment from pH 4 to 10 by the addition of 1.0 M solutions of NaOH or HCl. They were adjusted only prior to inoculation and even when the pH was altered during proliferation and amorpha-4,11-diene accumulation, they were not readjusted nor buffered during growth. The engineered yeasts were grown in 10 ml SC-Ura-Ade drop-out liquid medium at 30°C to OD_{600} between 1 and 2. Then, the 1 ml culture was inoculated into 50 ml fresh YPD medium with different pH to continue cultivation for three to four days at 30°C. All flasks also contained 2 ml dodecane. 200 µl dodecane layer was sampled and 1 µl out of the 200 µl dodecane was quantified for amorpha-4,11-diene production by GC-MS.

Statistical analysis

To evaluate the effect of carbon source, glucose concentration, pH and nitrogen source on amorpha-4,11-diene production in engineered yeasts with different mating types, the independent-samples T test analysis in SPSS programme was performed, and the significant difference of means were compared at $p < 0.05$.

RESULTS

Determination of amorpha-4,11-diene in engineered yeasts with different mating types

YPD medium with 1% yeast extract, 2% glucose and pH 8.0 was used to test for amorpha-4,11-diene production capacity on engineered yeasts with different mating types. Three positive clones of W303A[ADS] and W303B[ADS] were cultured and the dodecane layer were analyzed by GC-MS, respectively. The valencene standard curve was used for the quantification of amorpha-4,11-diene. The results show that positive clones all produced amorpha-4,11-diene (Figures 1 and 2). The amorpha-4,11-diene production of W303A[ADS] and W303B[ADS] was 12.43 ± 2.28 $mg \cdot L^{-1}$ (n = 3) and 12.61 ± 3.49 $mg \cdot L^{-1}$ (n = 3), respectively. The amorpha-4,11-diene production produced by engineered yeasts with different mating types had only a minor difference and the difference was not statistically significant (Figure 3).

Effect of carbon sources on the amorpha-4,11-diene production

As shown in Figure 4, glycerol, xylose and maltose were not good carbon sources in amorpha-4,11-diene production. On the other hand, sucrose, fructose, glucose and galactose could be better carbon sources in the YPD medium for amorpha-4,11-diene production. Among the carbon source tested, sucrose gave higher amorpha-4,11-diene yield in both engineered yeasts. When the YPD medium containing 2% glucose was used, the amorpha-4,11-diene production was not much different from sucrose and fructose. As sucrose and fructose are relatively expensive compounds and taking into account the cost of industrial application, glucose was used as the first choice of the carbon source. The engineered yeasts could not use xylose as carbon source and production of amorpha-4,11-diene in YPD medium with xylose as carbon source may be the result of 1 ml inoculation which contained glucose. The amorpha-4,11-diene production in W303A[ADS] was a little higher than that of W303B[ADS] when sucrose, fructose, glucose and galactose were used as carbon sources. The result, however, was reversed when maltose was used as carbon source. From these data, there was no statistically significant difference between the two malting types.

Effect of glucose concentrations on the amorpha-4,11-diene production

As illustrated in Figure 5, in the different concentrations tested, the changing trend of amorpha-4,11-diene production in both engineered yeasts was almost the same. In W303A[ADS], the amorpha-4,11-diene yields were raised following an increase of glucose concentrations from 0.4 to 2% and the amorpha-4,11-diene production level reached the maximum in YPD medium with 2% glucose concentration. The amorpha-4,11-diene yields declined with further increase of glucose concentrations from 2 to 4% in W303A[ADS]. In W303B[ADS], the highest amorpha-4,11-diene production was found in YPD medium with 3% glucose concentration and the yields decreased with further improvement to 4% in glucose concentrations. The amorpha-4,11-diene production of W303B[ADS] was higher than that of W303A[ADS] in YPD medium containing different glucose concentration from 0.4 to 4%. The yields differences, however, had no statistical significance.

Effect of media pH on the amorpha-4,11-diene production

The growth rates of the two engineered yeasts in the YPD medium at pH 4 to 10 were almost the same, which indicated a relative insensitivity of the engineered strains to a large range of initial pH values. As shown in Figure 6, the highest amorpha-4,11-diene production was found at pH 7.0 in both engineered yeast strains. This suggests that the optimum pH of amorpha-4,11-diene synthase is 7.0. These results are in consonance with others that were reported earlier (Picaud et al., 2005; Li et al., 2006). Yield differences of amorpha-4,11-diene existed between

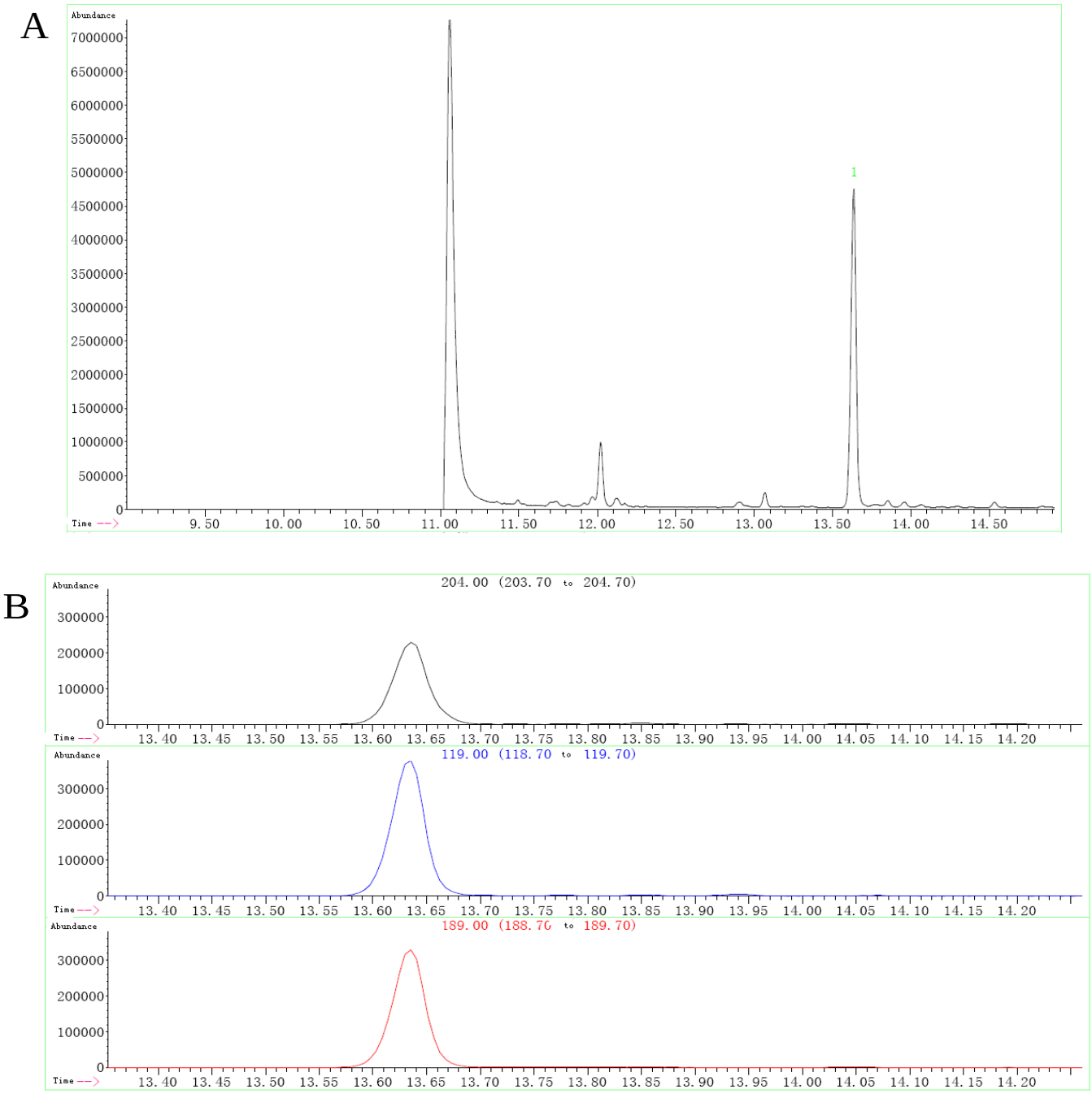

Figure 1. GC-MS identification of amorpha-4,11-diene produced by W303A[ADS]. A, The total ion chromatogram of W303A[ADS]; B, selected ion monitoring of W303A[ADS]; C, MS spectrum of the peak of retention time, 13.635 min.

the two engineered *S. cerevisiae* in the YPD medium with different origin pH. The yield differences had no statistical significance in this study.

Effect of different nitrogen sources on the amorpha-4,11-diene production

Five nitrogen sources were investigated to determine their effects on amorpha-4,11-diene production in W303A[ADS] and W303B[ADS]. In consideration of lowering the production costs for further industrial purposes, three inorganic nitrogen sources were also selected. Among the five organic or inorganic nitrogen sources, urea had the highest proportion of nitrogen content (46.7%), followed by NH_4NO_3 (35%), NH_4Cl (26.2%), $(NH_4)_2SO_4$ (21%) and yeast extract (10.0 to 12.5%).

Yeast extract is generally regarded as a suitable source

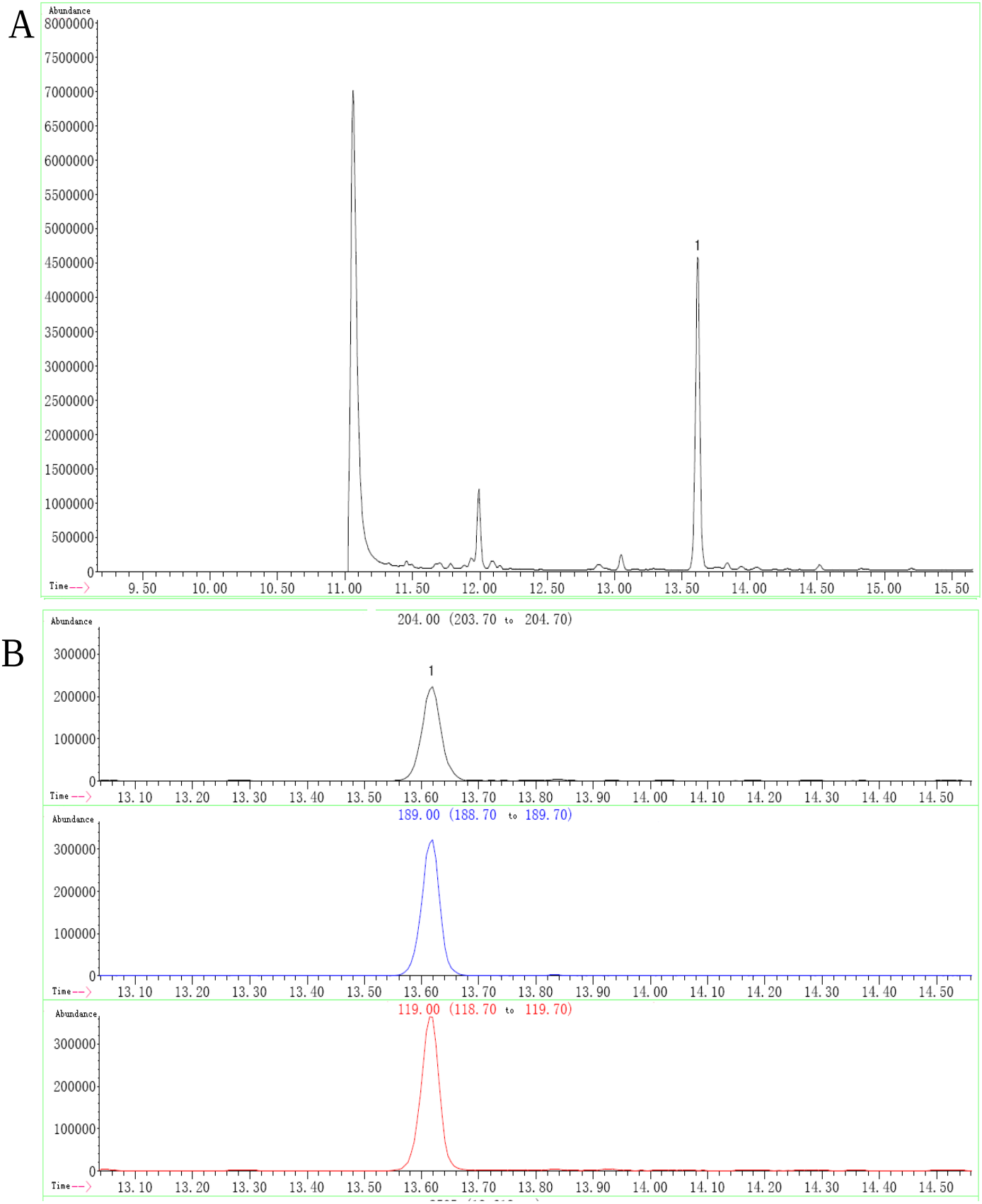

Figure 2. Continue.

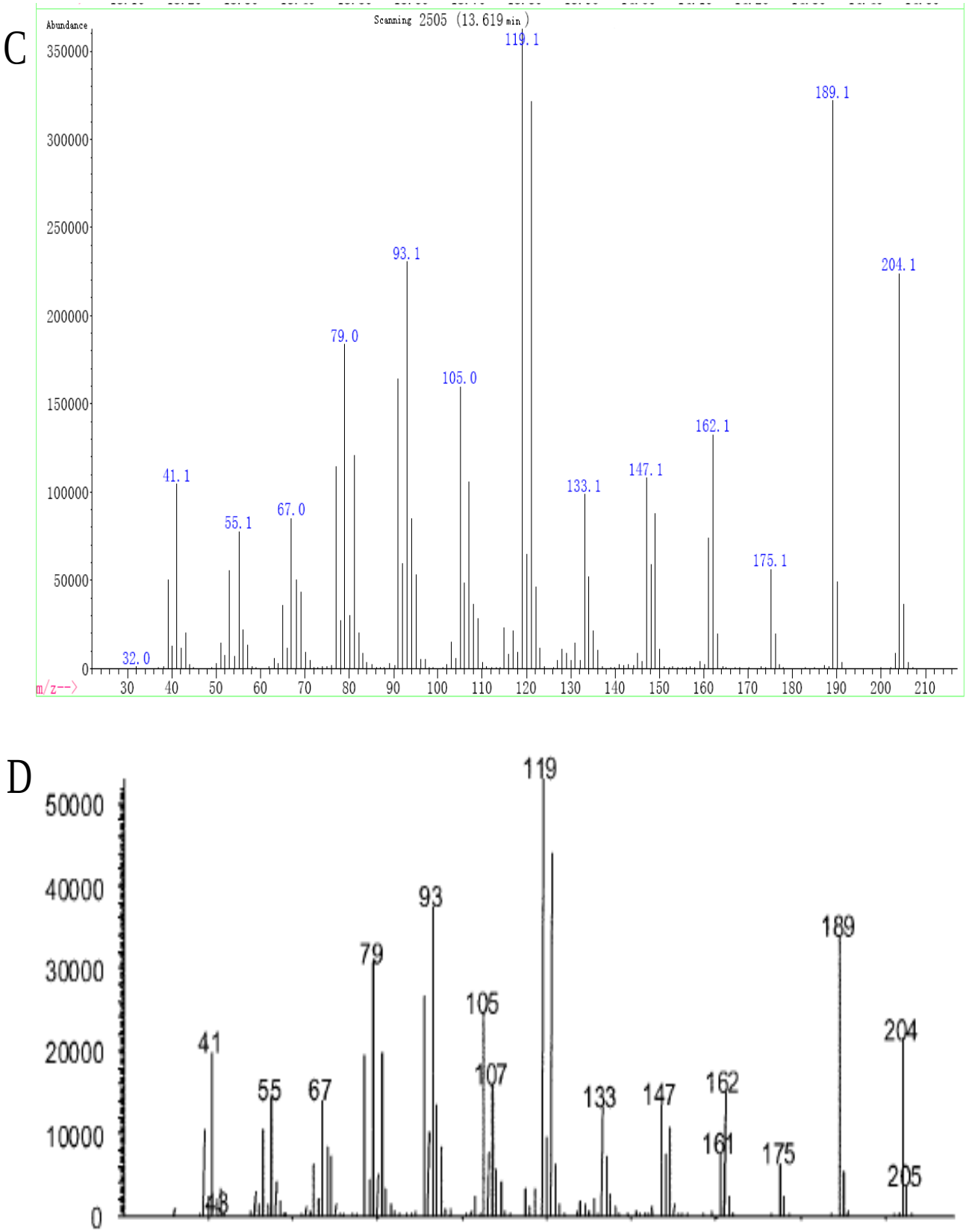

Figure 2. GC-MS identification of amorpha-4,11-diene produced by W303B[ADS]. A, The total ion chromatogram of W303B[ADS]; B, selected ion monitoring of W303B[ADS]; C, MS spectrum of the peak of retention time 13.619 min; D, MS spectrum of authentic amorpha-4,11-diene (Wallaart et al., 2001).

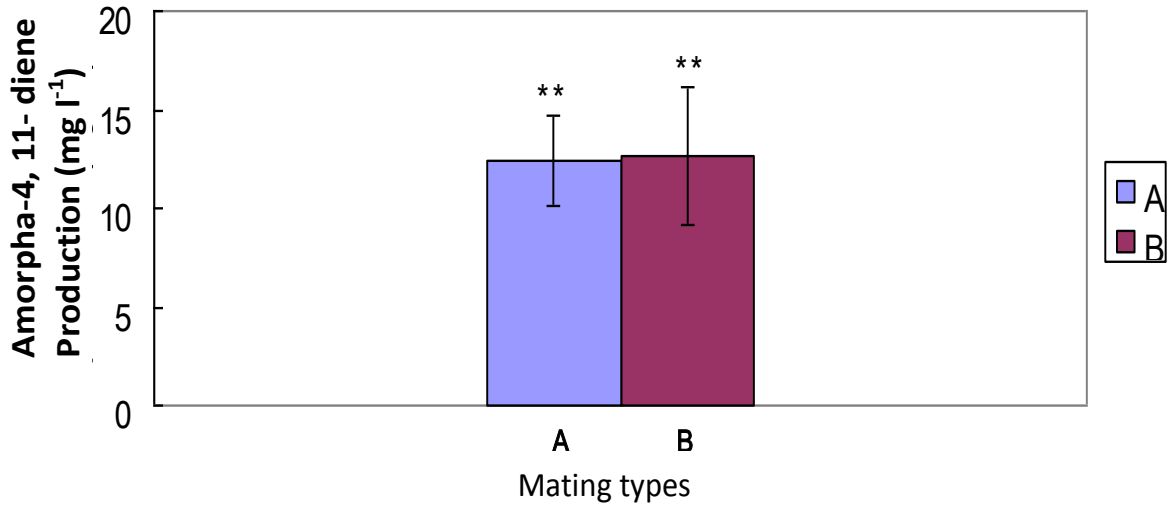

Figure 3. Amorpha-4,11-diene production by engineered yeasts with different mating types. A,Yeast strain W303-1A;B,Yeast strain W303-1B; **, No significant difference.

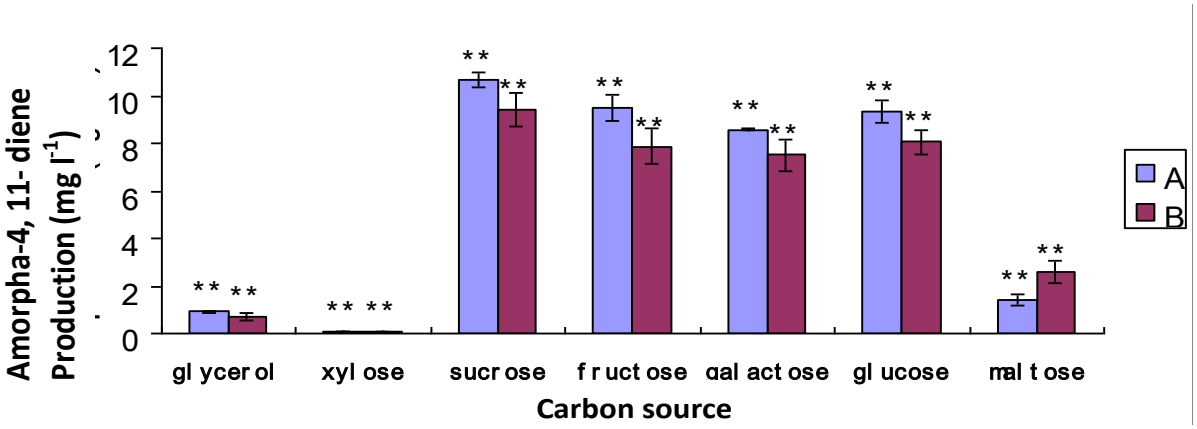

Figure 4. Effect of carbon on the amorpha-4,11-diene production. A,Yeast strain W303-1A;B,Yeast strain W303-1B; **, No significant difference.

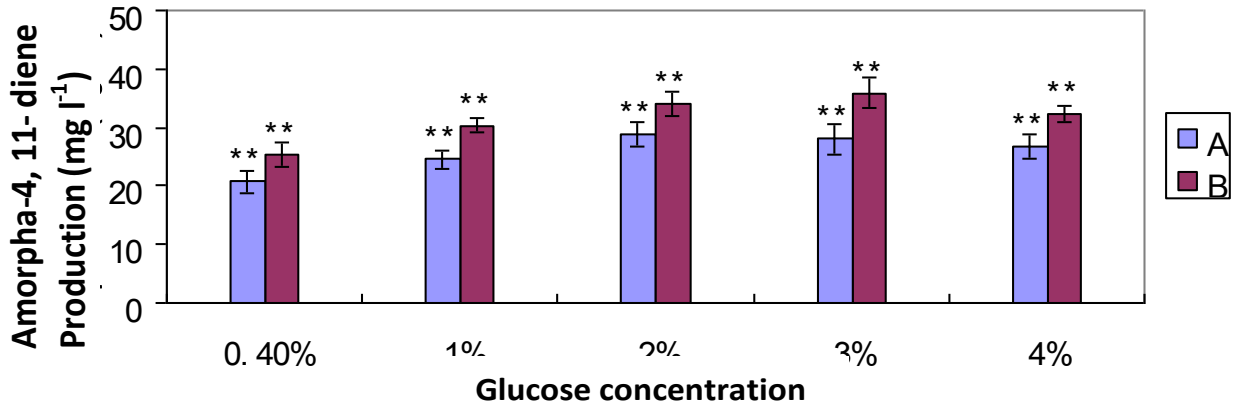

Figure 5. Effect of glucose concentration on amorpha-4,11-diene production. A,Yeast strain W303-1A;B,Yeast strain W303-1B; **, No significance difference.

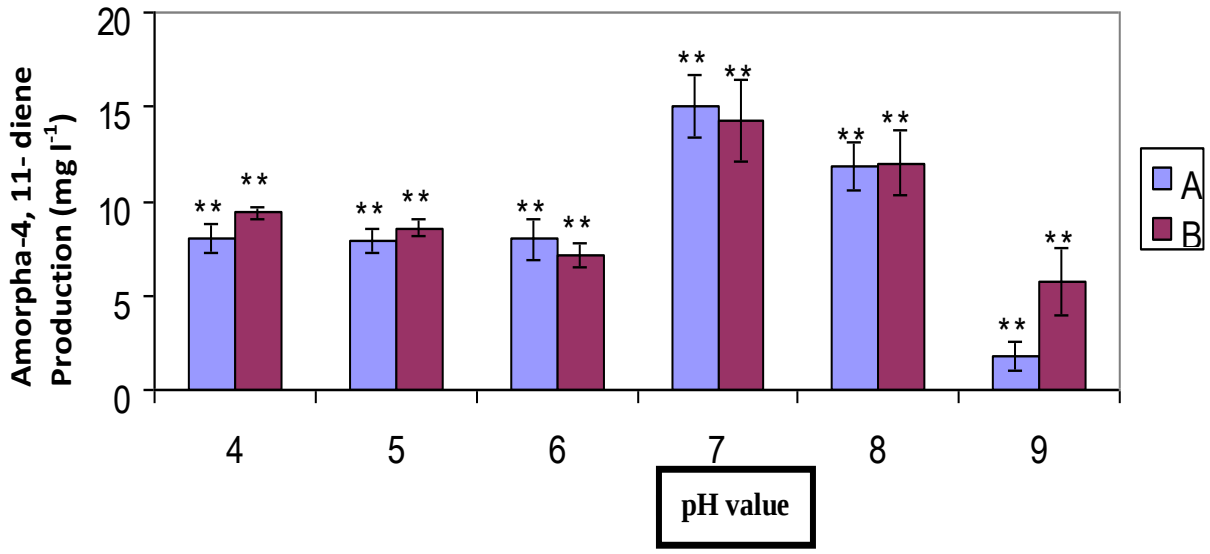

Figure 6. Effect of pH on amorpha-4,11-diene production. A,Yeast strain W303-1A;B,Yeast strain W303-1B; **, No significant difference.

of nitrogen and growth factors. As seen in Figure 7, the highest amorpha-4,11-diene production levels in the different mating types were obtained when 10 $g \cdot l^{-1}$ yeast extract was added to YPD medium. A slight difference in the amount of amorpha-4,11-diene production between W303B[ADS] and W303A[ADS] with different nitrogen sources mentioned could be observed, but was not statistically significant (Figure 7).

DISCUSSION

Using engineered microbial system to produce sesquiterpenes would provide an inexpensive and environmentally benign method different from synthetic routes or extraction from natural sources. In these systems, the yeast *S. cerevisiae* with sterol biosynthesis pathway is an attractive expression host for sesquiterpene production. *S. cerevisiae* exists in two haploid mating types, a and α mating types. Both haploid types were selected to construct engineered yeasts that can produce artemisinin precursors such as amorpha-4,11-diene, artemisinic acid and so on. Lindahl et al. (2006) used yeast with a mating type a to produce amorpha-4,11-diene. Ro et al. (2006), however, acquired amorpha-4,11-diene and artemisinic acid by the use of engineered yeasts with α mating type. In metabolic engineering, it is always important to identify the kind of mating types that would be appropriate to be the parent strain for further research and application. In this report, W303-1A and W303-1B were used to investigate whether mating type influences foreign sesquiterpene production. The GC-MS results show that engineered yeasts with different mating types produced amorpha-4,11-diene at the same level during a similar culture period. These results are suggestive that mating types showed no effect on amorpha-4,11-diene production, which are not consistent with the result reported by Jackson et al. (2003). The authors reported that FPP is more accessible in a strain for diversion to foreign sesquiterpene biosynthesis. The yeast cells with a mating type may normally biosynthesize more FPP as starting material for mating factor prenylation or may less rigorously channel FPP to sterol biosynthesis (Jackson et al., 2003).

For further investigation, the effects of four growth variables (carbon source, glucose concentration, nitrogen source and pH) on the fermentative production of amorpha-4,11-diene by engineered yeasts with different mating types were also studied. All data from this paper are an indication that no statistically significant differences in amorpha-4,11-diene production were observed in the study between both mating types of engineered yeasts. Our results suggest that both a and α types can be used as the parent strain for engineered yeast to produce amorpha-4,11-diene and other sesquiterpenes.

Most terpene synthases generate multiple products from the prenyl diphosphate substrate (Mercke et al., 1999; Picaud et al., 2005). The amorpha-4,11-diene synthase is not an exception (Mercke et al., 2000; Picaud et al., 2005; Yu et al., 2008). In this study, the amorpha-4,11-diene synthase produced ten more products as demonstrated in Figure 8. It is also suggested that amorpha-4,11-diene is not the only product in engineered yeasts of different mating types in this study. It is necessary to optimize the function of amorpha-4,11-diene synthase with application of conventional protein engineering such as directed evolution and rational and

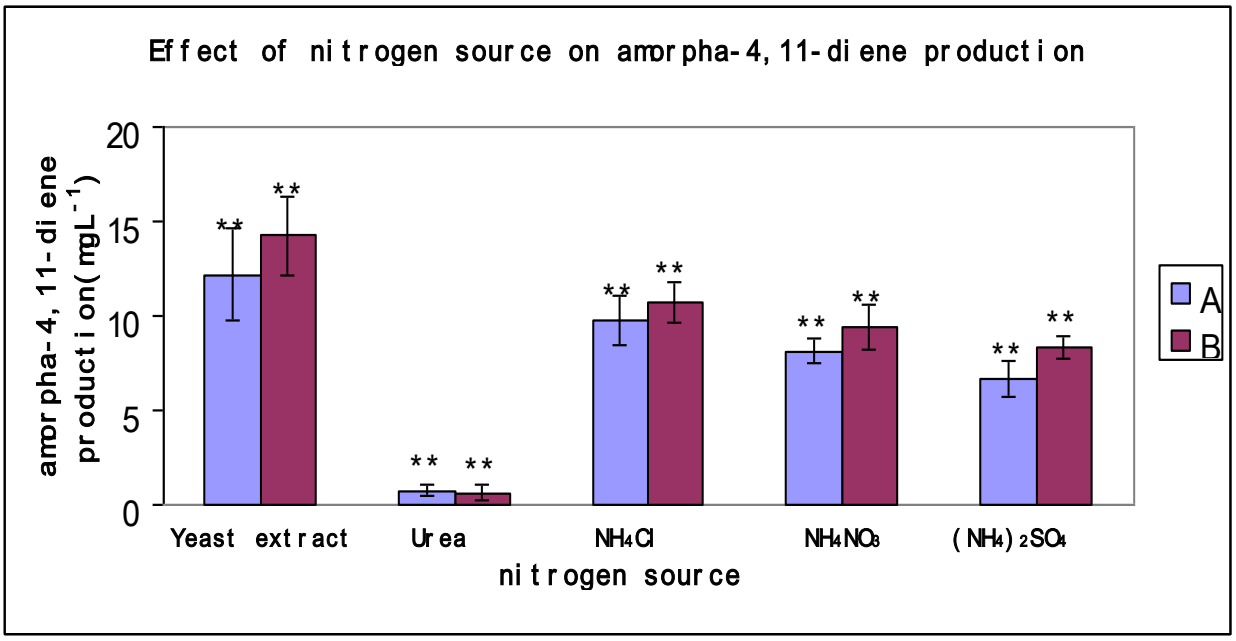

Figure 7. Effect of nitrogen source on amorpha-4,11-diene production. A,Yeast strain W303-1A;B,Yeast strain W303-1B; **, No significant difference.

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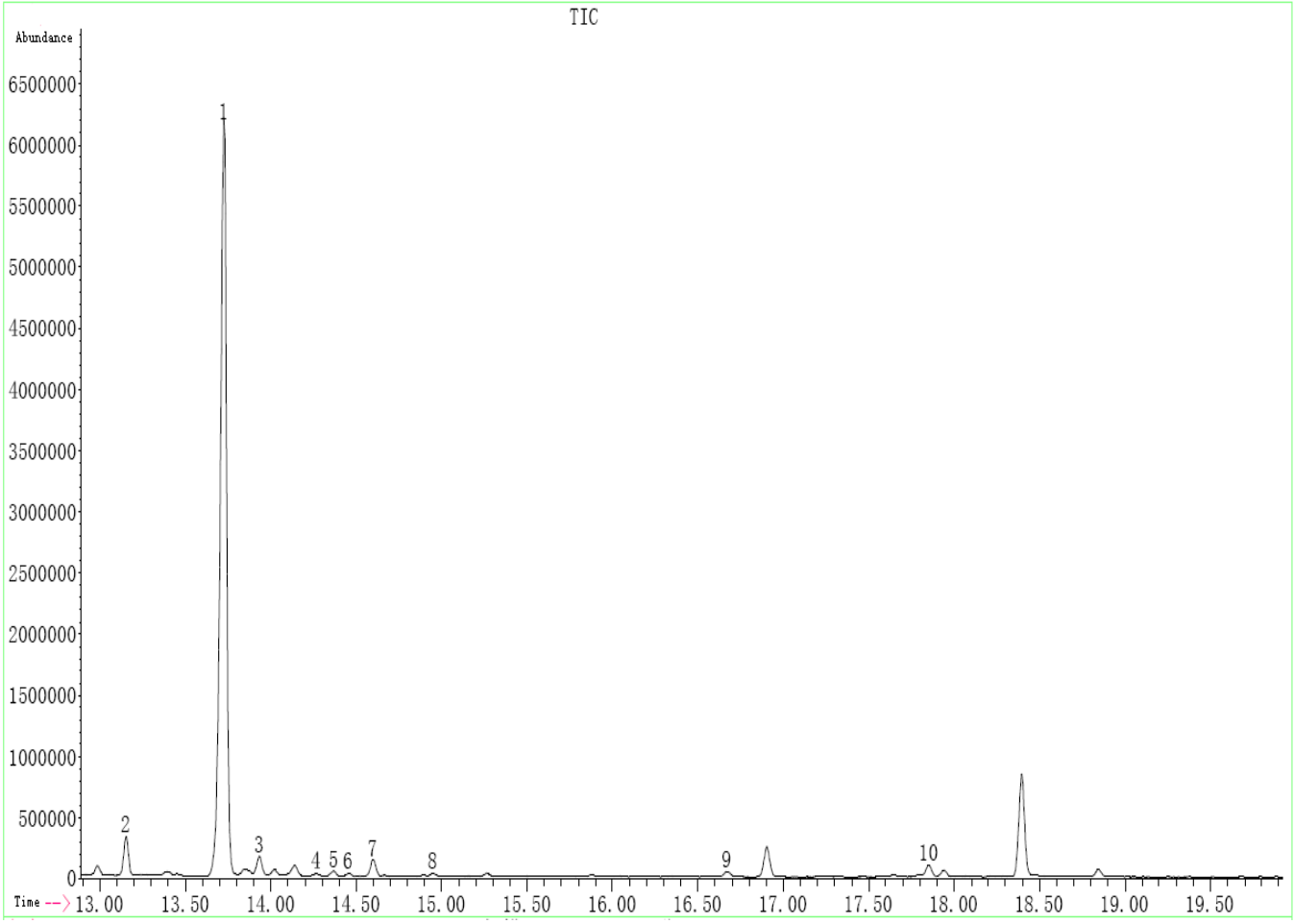

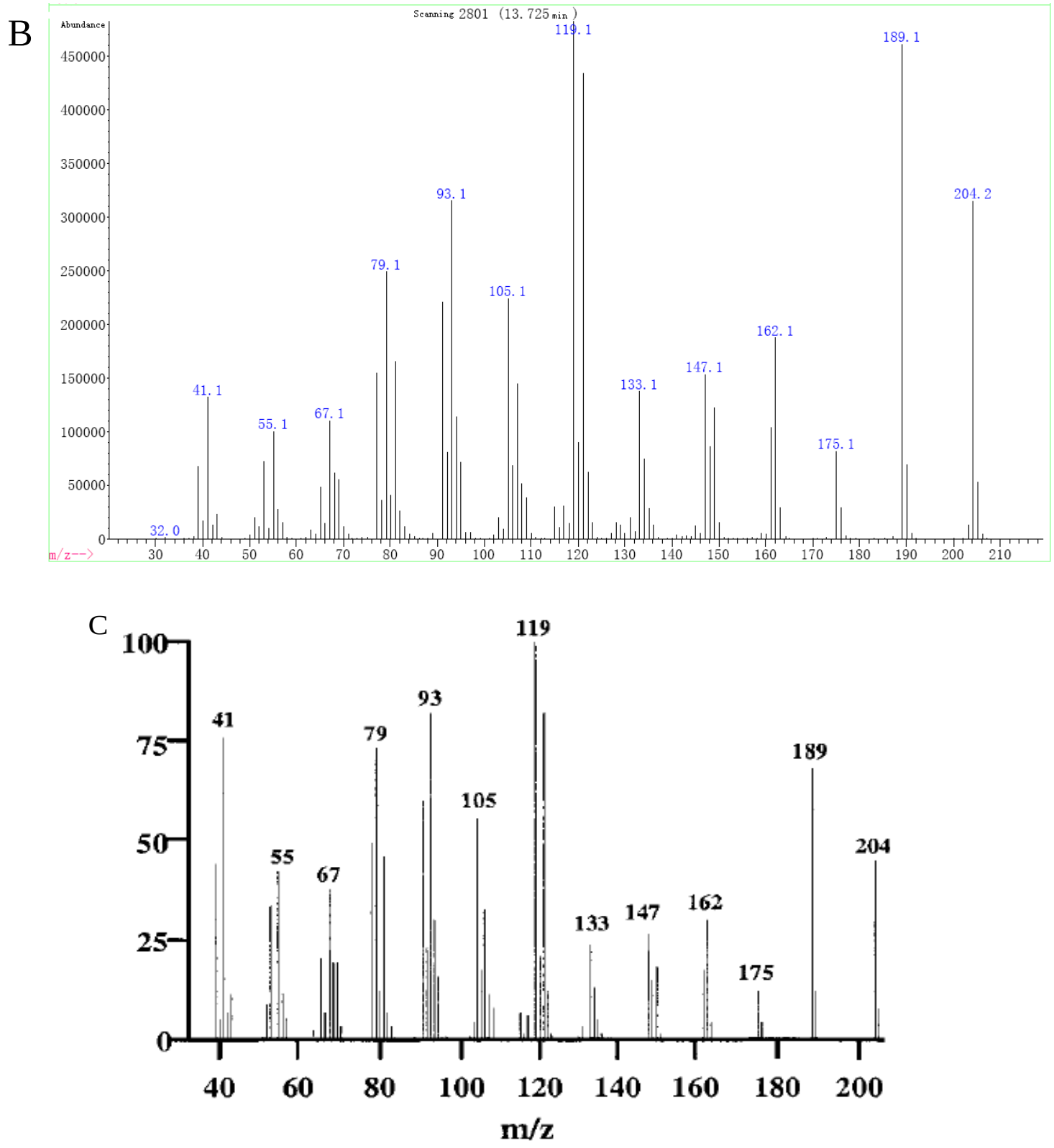

Figure 8. GC–MS analysis of the sesquiterpene product of W303B[ADS]. (A), Total ion chromatogram of sesquiterpene products generated from FDP by W303B[ADS]. The numbered peaks denote the sesquiterpenes: amorpha-4,11-diene; (1), (E)-β-farnesene (2), γ-humulene (3), unknown (4), unknown (5), unknown (6), β-sesquiphellandrene (7), longifolene (8), amorpha-4-en-11-ol (9), α-bisabolol (10); (B), Mass spectrum of the major biosynthetic product (peak 1); (C), Mass spectrum of authentic amorpha-4,11-diene (Mercke et al., 2000).

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China (30701061), National High-Tech Research and Development Plan (2007AA021501) and Beijing Municipal Natural Science Foundation (7082063).

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