

# **Mating of two laboratory *Saccharomyces cerevisiae* strains resulted in enhanced production of 2-phenylethanol by biotransformation of L-phenylalanine**

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**Running title** Diploid *S. cerevisiae* strain with enhanced 2-phenylethanol production

## **ABSTRACT**

2-phenylethanol (2-PE) is an aromatic alcohol with a rosy scent, which is widely used in the food, fragrance and cosmetic industries. Promising sources of natural 2-PE are microorganisms, especially yeasts, which can produce 2-PE by biosynthesis and biotransformation. Thus the first challenging goal to develop biotechnological production of 2-PE is searching for highly productive yeast strains. In the present work, five laboratory *Saccharomyces cerevisiae* strains were tested for the production of 2-PE. Thereafter, two of them were hybridized by a mating procedure and as a result, a new diploid, *S. cerevisiae* AM1-d, was selected. Within the 72 hours batch culture in a medium containing 5 g/l L-phenylalanine AM1-d produced 3.83 g/l 2-PE in a shaking flask. In this way, we managed to select the diploid *S. cerevisiae* AM1-d strain, showing a 3- and 5-fold increase in 2-PE production, in comparison to parental strains. Remarkable, the enhanced production of 2-PE by the hybrid of two yeast laboratory strains was demonstrated here for the first time.

**Key Words** 2-phenylethanol, Yeast, Diploid, Biotransformation, Biotechnology

## Introduction

2-Phenylethanol (2-PE) is an aromatic alcohol with a scent identifiable with roses. It can be found in the essential oils of many flowers and plants. It is used in the food, fragrance and cosmetic industries. There are three possible methods for obtaining 2-PE: chemical synthesis, extraction from rose petals and microbial production (biosynthesis or biotransformation); but only extraction from plants and microbial route provide a “natural” product according to US and European legislation [Serra et al., 2005]. 2-PE obtained from natural sources is much more expensive [Cui et al., 2011] and although its contribution in worlds market is not significant today, growing demand for natural products opens up possibilities for the utilization of alternative methods. Microorganisms that have an ability to produce 2-PE are bacteria, such as *Microbacterium sp.* and *Brevibacterium linens*, and fungi, such as *Aspergillus niger* and *Phellinus ignarius* [Etschmann et al., 2002]. However, yeasts *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are the most common organisms in this application [Garavaglia et al., 2007; Stark et al., 2003]. In yeast cells, 2-PE can be synthesized *de novo* or produced by biotransformation of L-phenylalanine (L-Phe) through the Ehrlich pathway [Eshkhol et al., 2009; Fabre et al., 1998]. An appropriate amount of L-Phe and diminished additional nitrogen sources are required for the predomination of the Ehrlich pathway [Fabre et al., 1998]. Furthermore, the productivity of 2-PE is associated with the type of yeast [Seo and Ahn, 2003], yeast growth, carbon source and media components [Fabre et al., 1998]. Since yeasts synthesize and secrete 2-PE to broth at a low level, because of its toxicity to the cells [Stark et al., 2003], the main challenge is to find a strain that will be able to tolerate high 2-PE concentrations and exhibit satisfactory production yield. Recent reports show that both *de novo* synthesis and the production of 2-PE *via* L-Phe biotransformation can be significantly increased by applying

metabolic engineering [Kim B et al., 2014; Kim T-Y et al., 2014; Romagnoli et al., 2015; Yin et al., 2015]. However, non-GMO strategies, because of complicated legislation and consumers negative attitude to GMO, are highly recommended and previously have been successfully applied to industrial yeast strains, including improvement of ethanol tolerance [Stanley et al., 2010], copper resistance [Adamo et al., 2012] or aroma production [Steensels et al., 2014]. Recent studies have also shown that hybrid strains often exhibit a heterotic phenotype in regards to compounds in the Ehrlich pathway [Krogerus et al., 2015, 2016]. Hence, it is likely that enhanced 2-phenylethanol production can also be achieved through hybridization.

In this study, we tested five haploid *S. cerevisiae* laboratory strains for their ability to produce 2-PE by synthesis *de novo* and by conversion of L-Phe. Then two of them were crossed by a mating procedure. In effect, a new non-GMO diploid *S. cerevisiae* AM1-d strain was achieved, which seems to be a good candidate for 2-PE production. It produces 3.83 g/l 2-PE with productivity rate of 53.19 mg 2-PE/l/h in a non-optimized batch culture in a shaking flask. Comparing to the previously reported 2-PE concentrations achieved under similar growth conditions by *S. cerevisiae* strains, e.g. Giv2009 (2.1 g/l 2-PE), Ye9-612 (0.85 g/l 2-PE) or CWY132 (1.39 g/l 2-PE and 3.52 g/l 2-PE after medium optimization) [Cui et al., 2010; Eshkol et al., 2009; Stark et al., 2002], the values obtained with AM1-d are considerably higher.

## Results and Discussion

### Characterization of laboratory haploid *S. cerevisiae* strains for 2-PE production

A number of yeasts can produce 2-PE from L-Phe through the Ehrlich pathway. However, a low yield and intolerance to high 2-PE concentration are the key problems that may restrict the development of a commercial cultivation process for the biotransformation of 2-PE. Because of the increasing interest in natural 2-PE, considerable efforts have been made to overcome economically low productivity. These methods include mainly: selection, mutation and genetic

modification of strains [Hua and Xu, 2011; Kim et al., 2014] as well as the optimization of the medium composition and the cultivation conditions [Eshkhol et al., 2009].

In this study, we first tested whether mutations affecting glucose metabolism may also influence 2-PE production. We have been studying, in our laboratory, the mechanisms controlling glucose metabolism, and have observed that the *S. cerevisiae* strains JM1-4D and MJ15-9C, which are affected in polymerase III RNA activity, express various intensities of the fermentation process - ethanol production [partly reported in Mierzejewska and Chreptowicz, 2016]. Since the inhibitory effects of ethanol on L-Phe bioconversion are known [Wittman et al., 2002; Stark et al., 2003] we were interested in finding out whether diminished fermentation in the MJ15-9C mutant strain could increase 2-PE production. We also wanted to determine if enhanced fermentation in JM1-4D may have a synergistic inhibitory effect on 2-PE production. Therefore, we tested the MB159-4D wild type strain and its two derivatives, JM1-4D and MJ15-9C, for the production of 2-PE using complete medium YPD for synthesis *de novo* and known medium 2 dedicated for the bioconversion of L-Phe to 2-PE [Huang et al., 2000] in a 48 h batch culture. We observed that these strains were able to produce 2-PE, however at low ranges from  $0.15\pm0.01$  to  $0.18\pm0.03$  g/l 2-PE and from  $0.08\pm0.02$  to  $0.53\pm0.02$  g/l 2-PE in YPD and medium 2 after 48 h, respectively (fig. 1a). While we did not notice significant differences between the strains in the level of 2-PE synthetized *de novo*, variations in 2-PE production by conversion of L-Phe were observed. The concentration of 2-PE was 2 and 6 times lower in JM1-4D and MJ15-9C than in MB159-4D, with  $0.27\pm0.04$  g/l 2-PE and  $0.08\pm0.02$  g/l vs  $0.53\pm0.02$  g/l 2-PE in medium 2, respectively (fig. 1a). Therefore, as we suspected, enhanced fermentative metabolism in JM1-4D might influence 2-PE production. However, comparing the OD<sub>600</sub> values achieved by JM1-4D and MB159-4D, 2.21 vs 5.08, it is also likely that the diminished growth in medium 2 might cause the decreased 2-PE production by JM1-4D. Contrary to our assumptions, the lowest concentration of 2-PE was in the culture of strain MJ15-9C, which was

$0.08 \pm 0.02$  g/l after 48 h. It is likely that the MJ15-9C strain is not oriented to 2-PE biosynthesis, because of its robustly slower growth (fig. 1a).

Next, we taken two additional haploid *S. cerevisiae* strains, AT12-1C and AB1-4A, collected in our laboratory and tested them for 2-PE production. Because they were only single auxotrophs, we could use two additional known media dedicated to L-Phe bioconversion to 2-PE, medium 3 and NEO [Eshkol et al., 2009; Etschmann et al., 2004]. Moreover, we prolonged incubation time to 72 hours, according to the previous studies on *S. cerevisiae* Ye9-612 strain producing 2-PE in medium NEO [Eshkol et al., 2009]. We observed that 2-PE production with AB1-4A and AT12-1C varies depending on the medium used (fig. 1b). For AB1-4A, the highest 2-PE concentration was noted after 72 h incubation in medium NEO, at  $1.02 \pm 0.05$  g/l (with  $Y=34\%$  and  $P= 14.10$  mg/l/h), and in medium 3, at  $0.97 \pm 0.04$  g/l (with  $Y=15\%$  and  $P=13.42$  mg/l/h), whereas for AT12-1C, medium 3 was the best one for 2-PE production, and a  $1.22 \pm 0.06$  g/l concentration (with  $Y=18\%$  and  $P=16.94$  mg/l/h) was achieved. The titter of 2-PE achieved by AB1-4A in NEO medium is slightly higher than that one obtained with Ye9-612,  $1.02$  vs  $0.85$  g/l [Eshkol et al., 2009]. When comparing the level of 2-PE in medium 2, there was a significant difference between the tested strains, and during the 72 h culture, AB1-4A produced almost five times more 2-PE than AT12-1C, with concentrations of  $0.77 \pm 0.03$  g/l vs  $0.16 \pm 0.01$  g/l 2-PE. We observed that the AB1-4A strain synthesizes *de novo* 2-PE in YPD more efficiently than AT12-1C, with concentrations of  $0.18 \pm 0.02$  g/l vs  $0.07 \pm 0.01$  g/l 2-PE. The lower 2-PE production by AT12-1C strain in medium 2 and YPD might be caused by the slower growth, since AT12-1C achieved only the OD<sub>600</sub> values of 2.11 and 6.46, respectively, while AB1-4A 5.68 and 11.56. However, we noticed also that despite the diminished growth of both AT12-1C and AB1-4A, the strains reached high concentrations of 1.22 and 0.97 g/l 2-PE. Hence, it seems that correlation between the biomass formation and 2-PE production depends on the medium.

## **Selection of diploid *S. cerevisiae* AM1-d and testing for 2-PE production in a batch culture**

Yeast strains used in the industry are often diploid or polyploid [Benitez et al., 1996; Yamada et al., 2010], due to their higher physiological activity. Thus, we fused the single auxotrophic haploids AT12-1C and AB1-4A by the mating process and then selected a prototrophic diploid strain (fig. 2), named *S. cerevisiae* AM1-d. Afterwards, we tested the AM1-d strain for 2-PE production using the same media, YPD, NEO, medium 2 and 3, and a 72 h batch culture as in case of parental strains (fig. 3a). During the cultivation, except the growth monitoring, we also measured the L-Phe consumption (fig. 3b, 3c). We observed that the diploid strain significantly enhances 2-PE production (fig. 3a) and shows an increase in production yield, comparing to parental strains (fig. 1b). Besides, AM1-d grows visibly better in media dedicated for 2-PE production (fig. 3b) than parental strains (fig. 1b) and reaches the 2-4 fold higher values of OD<sub>600</sub>. The AM1-d strain synthesized 0.25±0.02 g/l 2-PE *de novo* after 72 h incubation in YPD. In medium NEO, the estimated 2-PE concentration was 3.07±0.14 g/l 2-PE with productivity of 42.64 mg 2-PE/l/h, whereas in medium 2 it was 0.93±0.04 g/l 2-PE with *Y*=126% and *P*=12.92 mg 2-PE/l/h. The biotransformation efficiency (*Y*) above 100% achieved in medium 2 means that a significant part of the produced 2-PE was synthetized *de novo*. The highest concentration of 2-PE was reached in medium 3, in which AM1-d produced 3.82±0.16 g/l 2-PE with *Y*=59% and *P*= 53.06 mg 2-PE/l/h. We found that *S. cerevisiae* AM1-d consumed 4.43±0.18 g from 9 g of L-Phe present in medium 3 (fig. 3b). Therefore, we prepared medium 8 with the decreased concentration of L-Phe to 5 g/l, and tested the AM1-d strain for the 2-PE production in this medium. Although the L-Phe concentration in medium 8 was 5 g/l it was enough to obtain the same 2-PE concentration as in medium 3, 3.83±0.16 g/l (with *P*=53.19 mg 2-PE/l/h), which contained 9 g/l L-Phe (fig. 3a). Thus, 2-PE production occurred with *Y*=104% in medium 8 and the yield of 2-phenylethanol slightly above 100% can be explained by the fact that 2-PE is also synthesized *de novo* not only through L-Phe biotransformation. Additionally, the parental

auxotrophic AT12-1C and AB1-4A strains were also checked for the production of 2-PE in the medium 8 supplemented with either adenine or histidine, respectively. After 72 h incubation, the AT12-1C and AB1-4A strains produced  $1.32 \pm 0.07$  (with  $P=18.33$  mg 2-PE/l/h and  $Y=36\%$ ) and  $0.740 \pm 0.09$  (with  $P=10.27$  mg 2-PE/l/h and  $Y=20\%$ ), respectively. Therefore, a 3-5 fold higher 2-PE concentration with diploid strain was achieved in comparison to parental strains. Then, we conducted further studies on the 72 h batch culture of the AM1-d grown in medium 8. We calculated the specific growth rate during logarithmic phase, which was  $\mu=0.29$  h<sup>-1</sup> and monitored the glucose consumption (fig. 4). Besides, we estimated 2-PE concentration in the fast growing culture and after entrance into the stationary phase. Our results indicate that 2-PE is produced by AM1-d strain in the exponential growth phase as well as in the stationary growth phase (fig. 4).

Presented results show that by using the mating procedure, we obtained a yeast strain that is one of the first strains to exhibit such good production characteristics in a batch culture [Fabre et al., 1998; Stark et al., 2002; Eshkhol et al., 2009; Wang et al., 2011]. Although a higher 2-PE concentration of 4.8 g/l in a batch culture in a shaking flask has been recently reported with the genetically engineered *S. cerevisiae* JHY315 strain, but it was produced from 10 g/l L-Phe after 195 hours, thus with  $P=24.62$  mg 2-PE/l/h and  $Y=65\%$  [Kim T-Y et al., 2014]. Therefore, from economical point of view the AM1-d strain seems to be a better producer of 2-PE. Noteworthy, the production rate achieved with AM1-d ( $P=53.19$  mg 2-PE/l/h) is even higher than the rate recently reported for genetically engineered *S. cerevisiae* SPO810 strain, which produced 2.61 g/l 2-PE after 60 hours ( $P=43.50$  mg 2-PE/l/h), however in a fed-batch process [Yin et al., 2015]. While in a batch culture in a shaking flask the SPO810 strain produced only 1.10 g/l 2-PE. Hence, a significant improvement in 2-PE production can be expected when applying a fed-batch cultivation for AM1-d strain. However, in the case of AM1-d it would be better to apply the fed-batch culture in conjunction with one of the ISPR techniques as in a

batch culture we obtained a boundary concentration for 2-PE of around 4 g/l, which is toxic for yeast and inhibits the production of 2-PE [Stark and von Stockar, 2003].

### **Effect of the presence of exogenous 2-PE on the growth and cellular morphology of diploid AM1-d and parental strains**

The diploid AM1-d strain shows an increased production of 2-PE in comparison to parental strains, AB1-4A and AT12-1C. It might be caused by a higher physiological activity, since it is commonly known that the diploid or polyploid yeast strains used in the industrial bioprocesses expresses the increased metabolic activity when comparing to haploids [Benitez et al., 1996; Krogerus et al., 2016], and enhanced resistance to the toxic 2-PE accumulating in broth. The AM1-d strain grows better than parental strains in media dedicated for L-Phe bioconversion to 2-PE, thus it might be the reason of increased 2-PE production. To elucidate whether the mating procedure have an influence on the susceptibility of yeast cells to the increased titer of 2-PE in broth, we conducted 48 h batch cultures of AM1-d and parental, AB1-4A and AT12-1C in YPD supplemented with exogenous 2-PE to the final concentration of 1, 2 or 3 g/l. We monitored the growth by measurement of OD<sub>600</sub> and the cellular morphology by microscopic observations (fig. 5). We noticed differences between the tested strains. For AM1-d the strongest resistance to the increased 2-PE concentration was observed, whereas AT12-1C was the most sensitive to the presence of 2-PE in broth. While all tested strains slowed down the growth after addition of exogenous 2-PE to broth, only the diploid AM1-d strain reached the similar OD<sub>600</sub> values to the control culture (without addition of 2-PE to broth) after 48 h incubation, respectively 8 and 7 at the titer of 2-PE 1, 2 and 3 g/l. Besides no significant differences in the growth, also the microscopic observation did not reveal any visible changes in the cellular morphology of AM1-d grown in the presence of 2-PE. In the case of haploids, AB1-4A and AT12-1C, we noticed that the concentration of 2-PE above 1 g/l inhibited the growth significantly and the final OD<sub>600</sub> values were two times lower at the titer of 2 g/l in comparison to control cultures, whereas at 3

g/l AT12-1C did not grow at all. The toxic effect of the presence of 2-PE in broth was also visible in the changed cellular morphology of parental strains. While the AB1-4A and AT12-1C cells grown in broth containing 2-PE enlarged the vacuoles, the AT12-1C cells were also irregular. Thus, the diploid AM1-d strain displays a stronger resistance to the exogenous 2-PE, which combined with the better growth, might be the main reason of the enhanced 2-PE production by AM1-d. Besides, other potential reasons for this heterotic phenotype cannot be excluded, e.g. enhanced activity of enzymes in the Ehrlich pathway, since through this pathway L-Phe is converted to 2-PE [Yamada et al. 2010; Krogerus et al. 2016]. It would be also interesting to compare, in further studies, several different diploid hybrids, constructed from different parent strains, in order to see how common this heterotic phenotype in regards to 2-PE synthesis is.

## Materials and Methods

### Yeast strains and media

The yeast strains *S. cerevisiae* used in this study are listed in Table 1. For 2-PE production five strains were tested, AT12-1C, AB1-4A, MB159-4D and two of its derivatives, JM1-4D, MJ15-9C and the diploid *S. cerevisiae* strain AM1-d, which was constructed in this study by the mating of the haploid auxotrophic strains AT12-1C and AB1-4A. Cells from single colonies of the auxotrophic strains AT12-1C (*ade*<sup>-</sup>) and AB1-4A (*his*<sup>-</sup>) were streaked and mixed with a sterile loop on a solid standard yeast extract peptone containing 2% (w/v) glucose (YPD) medium [Sherman, 2002]. Then the plate was incubated for 2 days at 30°C. Next, the yeasts grown on YPD were replica-plated on a solid standard synthetic minimal glucose (SD) medium [Sherman, 2002] and incubated for 2 days at 30°C to select a prototrophic diploid strain (ADE<sup>+</sup> HIS<sup>+</sup>). The obtained prototrophic strain, named AM1-d, was then subjected for DNA content analysis by flow cytometry (FACS) performed as described previously in Krol et al., 2015.

Comparing the DNA content of AM1-d to two standard *S. cerevisiae* strains YAS281 (1n) and YMS14 (2n) it was verified that AM1-d is a diploid strain. The AM1-d strain was deposited and given the accession number KKP 2055p in the International Depositary Authority Culture Collection of Industrial Microorganisms, IBPRS Poland.

The yeast strains were cultured in either YPD medium for routine procedures and *de novo* synthesis of 2-PE or media containing L-Phe, listed in Table 2, for the production of 2-PE *via* biotransformation of L-Phe. Ingredients used to prepare the media were purchased from BioShop Canada Inc. Burlington, Ontario; BIOCOPA Polska Ltd, Warsaw, Poland; Merck KGaA, Darmstadt, Germany and Laboratorios Conda, Madrid, Spain. All ingredients were suitable for cell culture.

### **Bioproduction of 2-PE in a batch culture**

Overnight cultures in YPD grown in Erlenmeyer flasks at 30°C were diluted to an initial optical density at a wavelength of 600 nm (OD<sub>600</sub>; spectrophotometer SP-830 PLUS Metertech) of 0.15 in a 80 ml respective medium in a 300 ml flask. To ensure the growth of AT12-1C and AB1-4A, media NEO and 3 were supplemented with either 4 µg/ml adenine or 4 µg/ml histidine, respectively. Batch cultures were incubated at 30°C with shaking at 200 rpm for 48 h (MB159-4D, JM1-4D and MJ15-9C strain) or 72 h (AT12-1C, AB1-4A and AM1-d) in triplicate. Growth was monitored by the measurement of OD<sub>600</sub>. The concentration of 2-PE and L-Phe in broth was estimated at indicated time points.

### **Yeast growth in the presence of exogenous 2-PE**

Overnight cultures of AT12-1C, AB1-4A and AM1-d in YPD were diluted to an initial OD<sub>600</sub> of 0.15 in a 50 ml fresh YPD medium in four 100 ml flasks and incubated at 30°C with shaking. When the cultures achieved OD<sub>600</sub> = 0.8 2-PE (Merck) was added to three flasks to a final concentration of 1 g/l, 2 g/l or 3 g/l and to the fourth flask 2-PE was not added (control culture).

The cultivation was carried out for 48 h and the growth was monitored by OD<sub>600</sub> measurement. Additionally to OD<sub>600</sub> measurements, microscopic observations were conducted at the 24 h and 48 h of incubation. The images of unstained cells were captured through a 100 x objective lens under bright field microscopy (Levenhuk D870T).

### **Analytical Methods**

Prior to determining 2-PE and L-Phe concentration in culture broth, 2 ml aliquots of culture were centrifuged for 10 min, at 2150 g and 4°C. The cellular pellets were discarded and supernatants were further analyzed. To determine the concentration of 2-PE and L-Phe, the culture supernatants were diluted in acetonitrile (dilution factor 2) and analyzed with high-performance liquid chromatography (HPLC). The Agilent Technologies 1200 chromatograph with a UV-VIS detector (Spectra System) and C18, 5 µm, 4.6 x 150 mm column (Zorba Eclipse XDB) were used. An isocratic method comprising water/acetonitrile (70/30) or water was applied for 2-PE or L-Phe analysis, respectively, with a flow of 1 mL/min. The 2-PE and L-Phe concentration was estimated at a wavelength of 210 nm. Glucose concentration was determined by Glucose Assay (BioMaxima, Lublin, Poland) according to the manufacturer's instructions. All analyses were performed in triplicate and data are presented as mean ± standard deviation. Statistical comparisons were performed between groups using Student's unpaired t-tests; p < 0.05 was the criterion for statistical significance.

### **Calculations**

According to the obtained data the production rate as  $P = \frac{\Delta C_{2-PE}}{\Delta t} [mg/l/h]$  and specific product yield as  $Y = \frac{\Delta C_{2-PE}}{\Delta C_{2-PE}^{theoretical}} 100\% [\%]$  were calculated; where  $\Delta C_{2-PE}$  is the ratio of the achieved concentration of the product (2-PE) and  $\Delta C_{2-PE}^{theoretical}$  is the maximum theoretical concentration of the product which can be achieved as a result of the biotransformation of L-Phe.

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## Conflict of Interest

The authors declare no conflict of interest associated with this research.

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**Table 1** *S. cerevisiae* strains used in this study

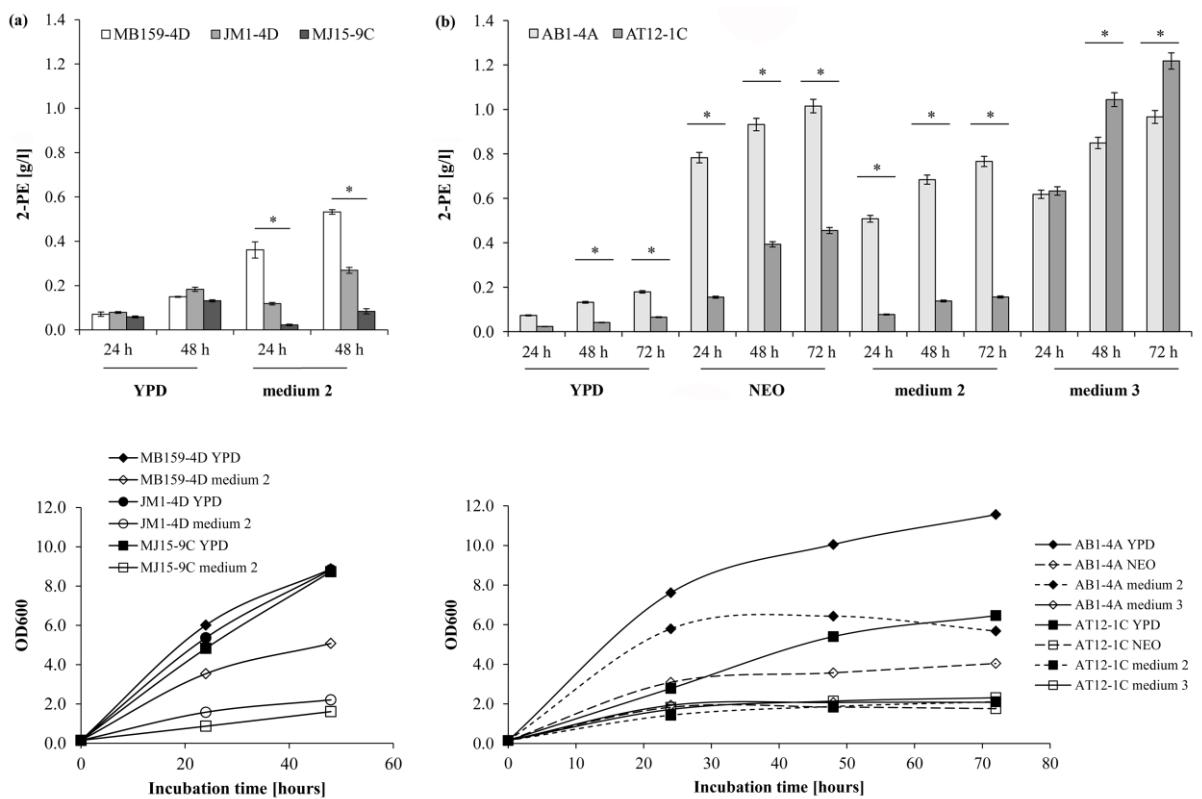
Strain	Genotype	Source
AB1-4A	<i>MATa his4</i>	IBB PAS*, Słonimski PP
AM1-d	<i>MATa/MATa ADE1/ade1 HIS4/ his4</i>	this study
AT12-1C	<i>MATa ade1</i>	IBB PAS*, Słonimski PP
JM1-4D	<i>MATa SUP11 ade2-1 ura3-1 leu2-3, 112 lys2-1 maf1Δ::URA3</i>	[Mierzejewska and Chreptowicz, 2016]
MB159-4D	<i>MATa SUP11 ade2-1 ura3-1 leu2-3, 112 lys2-1</i>	[Kwapisz et al., 2002]
MJ15-9C	<i>MATa SUP11 ade2-1 ura3-1 lys2-1 leu2-3, 112 rpc128-1007</i>	[Cieśla et al., 2007]
YAS281 (1n)	<i>MATa his3-1 leu2 met15 URA3</i>	[Alabrudzinska et al., 2011]
YMS14 (2n)	<i>MATa/MATa his3-1/his3-1 leu2/leu2 LYS2/lys2 met15/MET15 URA3/ura3 CAN1/can1::LEU2</i>	[Alabrudzinska et al., 2011]

\*Institute of Biochemistry and Biophysics Polish Academy of Sciences

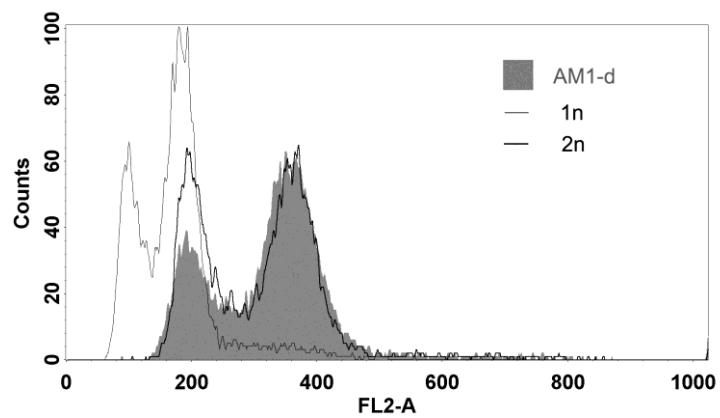
**Table 2** Media composition used in this study for 2-PE production *via* L-Phe biotransformation

Medium	Composition
NEO	20 g/l glucose, 4 g/l L-Phe, 4 g/l KH <sub>2</sub> PO <sub>4</sub> , 0.4 g/l MgSO <sub>4</sub> ·7 H <sub>2</sub> O, 1 g/l yeast extract, pH = 5.1 [Etschmann et al., 2004; Eshkol et al., 2009]
2	20 g/l glucose, 2.5 g/l yeast extract, 1 g/l L-Phe, pH = 6.1 [Huang et al., 2000]
3	22 g/l glucose, 8 g/l sucrose, 9 g/l L-Phe, 0.5 g/l MgSO <sub>4</sub> , 1.7 g/l YNB (yeast nitrogen base) without amino acids and ammonium sulfate, pH = 4.7 [Etschmann et al., 2004]
8	15 g/l glucose, 8 g/l sucrose, 5 g/l L-Phe, 0.5 g/l MgSO <sub>4</sub> , 1.7 g/l YNB without amino acids and ammonium sulfate, pH = 4.6

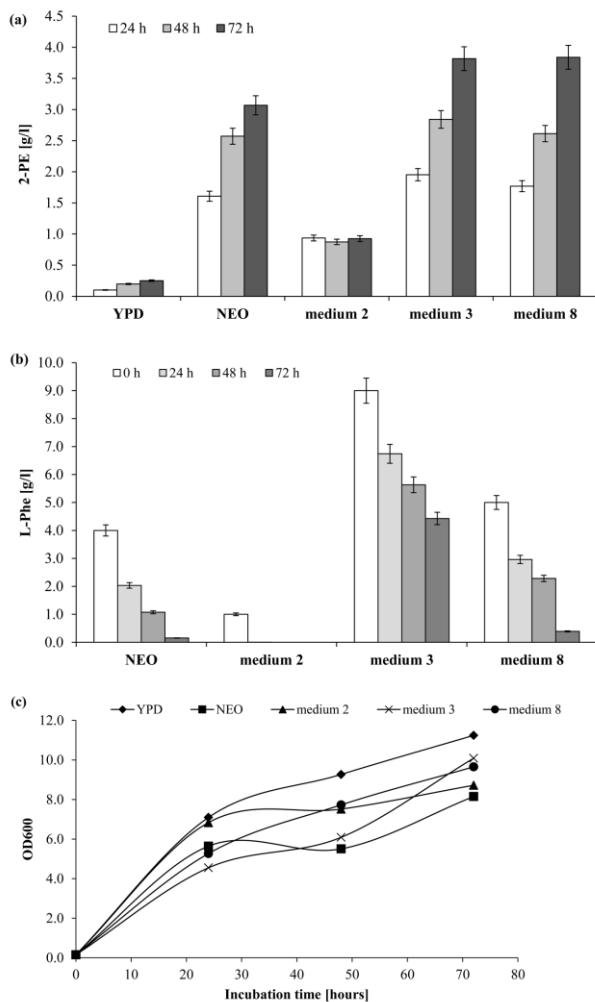
## Figures



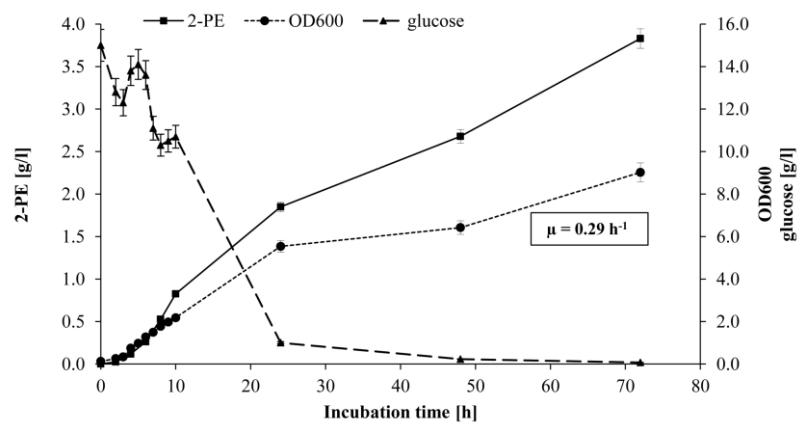
**Fig. 1.** 2-PE production by haploid *S. cerevisiae* laboratory strains in shaking flasks in various media. (a) MB159-4D, JM1-4D and MJ15-9C strains after 24 h and 48 h of incubation in YDP, medium 2. (b) AB1-4A and AT12-1C strains after 24 h, 48 h and 72 h of incubation in YPD, NEO, 2 and 3 medium. Analyses were performed in triplicate and data are presented as mean  $\pm$  SD, \*p < 0.05.



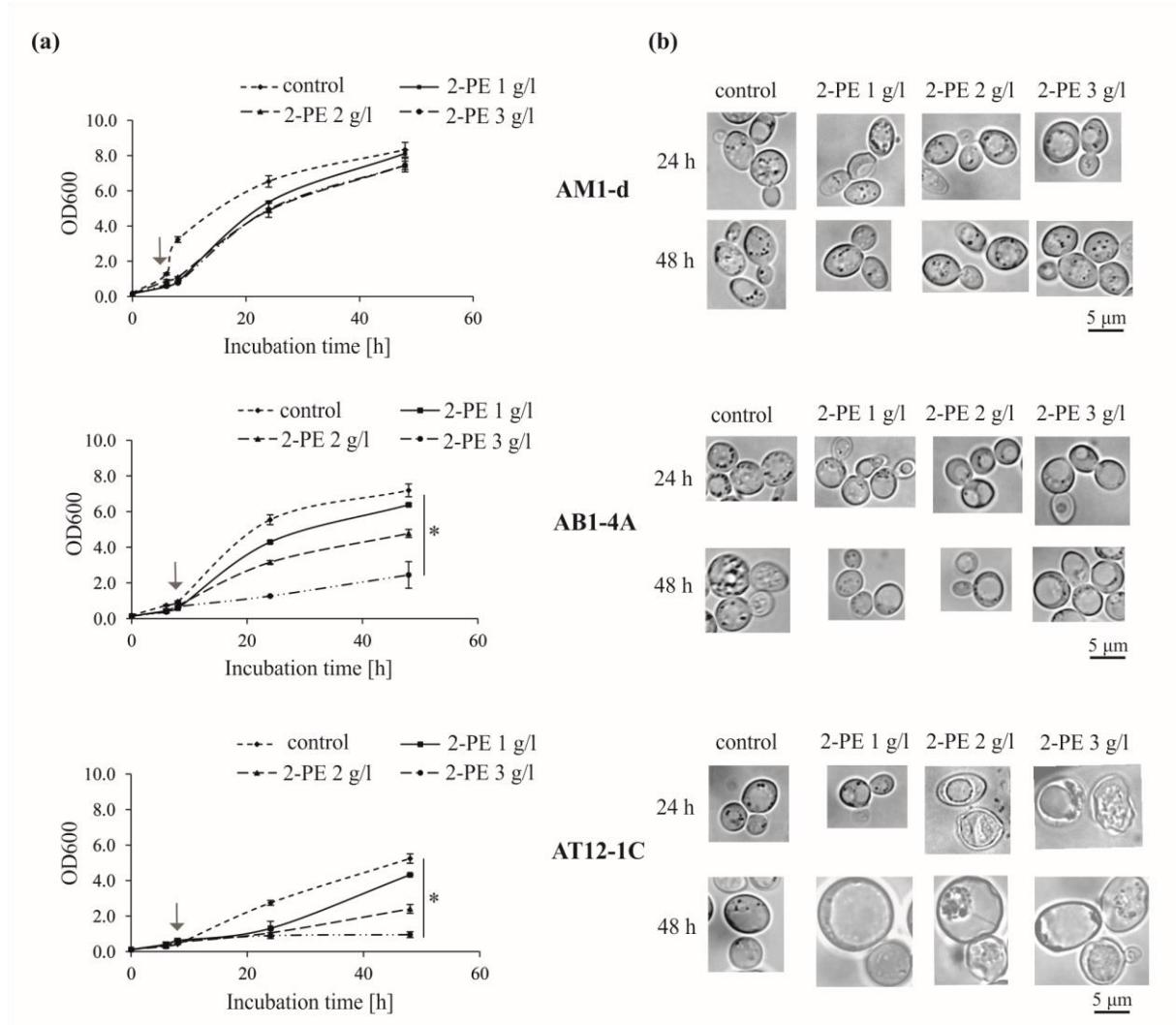
**Fig. 2.** DNA content analysis of AM1-d strain. Propidium iodide-stained cells from exponentially grown yeast cultures were analysed *via* FACS. Histograms were obtained for 10,000 cells per each strain. 1n and 2n strains served as DNA content controls for haploid and diploid strains, respectively.



**Fig. 3.** Production of 2-PE by AM1-d strain various media. (a) The concentration of 2-PE, (b) L-Phe consumption and (c) the OD<sub>600</sub> was measured in the batch cultures of *S. cerevisiae* AM1-d at indicated hours of incubation in YPD, NEO, medium 2, 3 and 8.



**Fig. 4.** Growth monitoring and 2-PE production in a 72 h batch culture of *S. cerevisiae* AM1-d in medium 8; OD<sub>600</sub> and the 2-PE and glucose concentration in broth were measured at indicated time points in triplicate.



**Fig. 5.** Effect of the presence of exogenous 2-PE on the growth (a) and cellular morphology (b) of AM1-d, AB1-4A and AT12-1C strains. For each strain four 48 h batch cultures in YPD were conducted. When the cultures reached OD<sub>600</sub> of 0.8, indicated by arrow, 2-PE was added to three cultures to a final concentration of 1 g/l, 2 g/l and 3 g/l, respectively. The fourth culture, not supplemented with 2-PE was as a control. Analyses were performed in triplicate and data are presented as mean  $\pm$  SD, \*p < 0.05.