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ORIGINAL ARTICLE

Production of 2-phenylethanol from L-phenylalanine by a stress tolerant *Saccharomyces cerevisiae* strain

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Abstract

Aims: Screening for a robust, stress tolerant *Saccharomyces cerevisiae* strain for production of 2-phenylethanol (PEA) from L-phenylalanine.
Methods and Results: *Saccharomyces cerevisiae* natural isolates that include thermotolerant and multi-stress resistant strains were screened for production of PEA. The strains were compared by their growth rate, maximum biomass dry weight and production yields, and it was shown that high biomass is required for obtaining high concentrations of PEA. One thermotolerant strain, Ye9-612, was the most efficient, and under optimal conditions produced 0·85 g l^{-1} PEA in shaking flasks, and 4·5 g l^{-1} in a fed-batch fermentation. Strain Ye9-612 was also found to be tolerant to high concentrations of PEA, and maintained high biomass dry weight in the presence of 2·5 g l^{-1} PEA.
Conclusions: We found a highly productive thermotolerant *S. cerevisiae* strain that is resistant to high concentrations of PEA.
Significance and Impact of the Study: PEA is inhibitory to growing yeast cells and therefore a robust strain is needed for excessive industrial production. This is the first description of such a stress tolerant strain, and the PEA concentration obtained in the fermentation is the highest reported to date.

Introduction

Extraction of flavours and fragrances from plants has been known and practiced for many decades. However, such natural extracts of pure compounds obtained by physical processes are very expensive due to low extraction yields (Brenna 2005; Serra *et al.* 2005). Biocatalytic processes may offer a cheaper alternative to natural production of flavours. Provided the substrate itself is a natural compound, then the bioconversion is considered a 'natural' route according to both US and European legislations (Fabre *et al.* 1997; Serra *et al.* 2005).

2-Phenylethanol (PEA) is an aromatic alcohol with a rose-like odour and occurs in many essential oils and fermented foods (Etschmann *et al.* 2004). An annual production of *c.* 7000 tons is generated by chemical processes, mainly by the Friedel–Craft reaction of ethylene oxide with benzene, or by hydrogenation of styrene oxide with Raney nickel as a catalyst (Etschmann *et al.* 2002). Both chemical synthetic methods involve toxic reagents and harsh conditions thereby creating by-products which reduce the quality of the final PEA. Removal of undesired contaminants is necessary before marketing of the product (Etschmann *et al.* 2002). A biotechnological route may provide 'natural' PEA at high purity by an environmentally friendly process.

It has been demonstrated previously, that various yeast strains produce PEA from L-phenylalanine by the action of three enzymes *via* the Ehrlich pathway (Fabre *et al.* 1997; Schrader *et al.* 2004). In this metabolic pathway, L-phenylalanine is transaminated to phenylpyruvate by a transaminase, decarboxylated to phenylacetaldehyde by a decarboxylase, and subsequently reduced to PEA by a dehydrogenase (Fig. 1). Relatively large quantities of L-phenylalanine, as well as the absence of other nitrogen sources, are required for the Ehrlich pathway to predominate (Etschmann *et al.* 2003, 2004). PEA production by yeast is growth associated (Fabre *et al.* 1998) and yeast-type dependent (Fabre *et al.* 1997; Seo and Ahn 2003). It has also been reported that the carbon source

and other media components, greatly influence the productivity (Fabre *et al.* 1998; Etschmann *et al.* 2004). The drawback of the biocatalytic process is the inhibition of yeast growth by the accumulating product, PEA (Fabre *et al.* 1998; Seo and Ahn 2003; Stark *et al.* 2003; Etschmann and Schrader 2006). Both aliphatic and aromatic alcohols are known inhibitors of yeast growth and reduce viability, moreover, they have a synergistic effect when combined (Ingram and Buttke 1984; Seward *et al.* 1996). Damage to cell membranes (Ingram and Buttke 1984), reduced uptake of glucose and amino acids (Lester 1965), and reduction in respiratory capacity (Stark *et al.* 2003) are some mechanisms involved in this inhibition phenomena. *In situ* product removal (ISPR) has been proven to be a useful approach for circumventing the problem of product inhibition (Stark and von Stockar 2003). Oleyl alcohol has been used to increase the PEA productivity of various yeast strain cultures by three- to eightfold (Fabre *et al.* 1997). von Stark *et al.* (2002) used oleic acid for a two-phase fed-batch culture of *Saccharomyces cerevisiae* Giv2009 to increase the concentration from 2·1 to 12·6 g l^{-1}. Recently, Etschmann and Schrader (2006) reported a high PEA concentration of 26·5 g l^{-1} by *Kluyveromyces marxianus* CBS 600 using polypropylene glycol 1200 as an extracting phase.

We have previously described the isolation and characterization of natural *S. cerevisiae* strains isolated from Mount Carmel National Park, Haifa, Israel (Katz-Ezov *et al.* 2006). Some of the progeny of these natural isolates showed a multi-stress resistance phenotype towards thermal, osmotic and oxidative stress (Nir *et al.* 2008). Here we describe the screening of various thermotolerant *S. cerevisiae* natural isolates for the production of PEA with the aim of finding a strain that will be both productive and tolerant to high concentrations of PEA. Such a robust micro-organism will be valuable for industrial production of PEA.

Materials and methods

Chemicals and media

PEA was purchased from Fluka and L-phenylalanine from Sigma (Sigma-Aldrich and Fluka, Rehovot, Israel). All materials used were of the highest purity available and were used without further purification. YPD medium contained (g l^{-1}): glucose – 20, peptone – 20, yeast extract – 10. NE medium contained (g l^{-1}): glucose – 5, L-phenylalanine – 4, KH_2PO_4 – 4, $MgSO_4 \cdot H_2O$ – 0·4, yeast extract – 1. For solid plates 20 g l^{-1} agar was added.

Yeast strains and growth conditions

The yeast strains used in this study include a natural isolate, designated Ye9 (diploid), that was isolated from 'Evolution Canyon' at Mount Carmel National Park in Haifa, Israel and its offspring: Ye9-633, Ye9-517, Ye9-602, Ye9-612, Ye9-531, Ye9-519, Ye9-511, Ye9-667, Ye9-668, Ye9-644, Ye9-523, Ye9-670, Ye9-561, Ye9-503, Ye9-518, Ye9-653, Ye9-639, Ye9-656, Ye9-660, Ye9-600, Ye9-666, Ye9-618, Ye9-526, Ye9-569, Ye9-532, Ye9-631, Ye9-654, Ye9-515 and Ye9-596 (Nir *et al.* 2008). The laboratory strains Y103 (*MAT* a, Lys1) and S288C (MATα *SUC2 mal mel gal2 CUP1*), were also employed in the study. All strains were maintained as glycerol stock solutions at −80°C in the collection of the Faculty of Biotechnology and Food Engineering at Technion, Haifa, Israel. The yeasts were routinely cultivated in YPD medium at 30°C with shaking at 250 rpm on an orbital shaker incubator.

Screen for thermotolerance

The screen included two steps: (i) 72 h growth at 40°C on solid YPD medium, and (ii) growth kinetics at 40°C. A cell culture grown overnight was diluted to 0·1–0·2 OD_{600} in YPD medium and grown at 40°C for 24 h with maximum shaking (level 4) in a multiplate reader Synergy HT (BioTek Instruments Inc., Winooski, VT, USA). Strains that grew on solid medium at 40°C and showed high growth rate and high cell density in the second step, were characterized as thermotolerant (Nir *et al.* 2008).

Screening of *S. cerevisiae* strains for the production of PEA from L-phenylalanine

Yeast cells were grown overnight on YPD plates at 30°C and inoculated into 25 ml of liquid NE medium at the denoted temperature with shaking at 250 rpm. After 48 h of growth, cell density ($OD_{600,}$ Novaspec Plus; Amersham Biosciences, Cambridge, UK) and concentration of PEA were measured [high performance liquid chromatography (HPLC)].

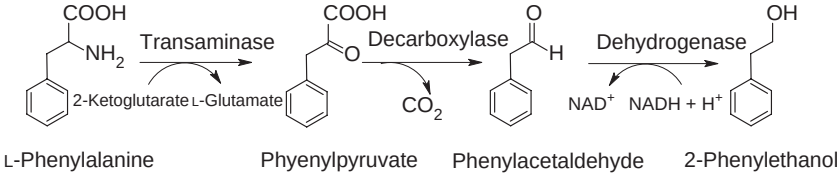

Figure 1 Ehrlich pathway of yeasts converting L-phenylalanine into PEA (Etschmann *et al.* 2002).

A standard curve was created in which optical density (at 600 nm) measurements were correlated to dry cell weight concentrations (gram dry weight per liter – gdw l^{-1}). Dry weight of biomass was determined in duplicate samples of 10 ml each by centrifuging 10 min at 13 400 ***g*** (4K15 centrifuge with 12172-H rotor; Sigma, Osteroid, Germany). The pellets were washed twice with distilled water by resuspension, and centrifuged again at the same conditions. The wet pellets were dried for 24 h in an oven at 90°C (MRC Ltd, Holon, Israel) after which they were weighed. Subsequently, all OD_{600} measurements were converted to dry cell weight concentrations using the determined correlation factor of 0·41 g l^{-1} OD^{-1}.

Growth and PEA production curves

Yeast cells were grown overnight on YPD plates at 30°C and a loop full of cells was used to inoculate 25 ml of liquid YPD. Following growth for 24 h at 30°C, the cell culture was diluted to an initial OD_{600} of 0·1 in a 60 ml NE medium and further incubated, at the denoted temperature with shaking at 250 rpm. Cell density and PEA concentrations were measured every 90 min.

Optimization of growth media for maximum production of PEA

Yeast cells were grown at 30°C as described above on NE media containing different glucose concentrations: 15, 20, 30 or 40 g l^{-1} (all the other components of the media remained the same). In a separate set of experiments the yeast cells were grown on media with the optimal glucose concentration (20 g l^{-1}), with different L-phenylalanine concentrations: 2, 4, 6 or 7 g l^{-1}. The optimal glucose and L-phenylalanine concentrations for production of PEA were chosen based on the maximum OD_{600} and final PEA concentration at each media composition.

Effect of PEA concentration on growth and biomass dry weight of selected strains

Yeast cells were grown at 30°C as described above, on NE media with 20 g l^{-1} glucose and 2 g l^{-1} $(NH_4)_2SO_4$ as the sole nitrogen source to prevent PEA production by the cells. Different PEA concentrations were added to the medium (0, 1, 1·5, 2, 2·5, 3, 4 g l^{-1}) and the yeast were grown for 24 h. The cell density was measured every 90 min.

Fed-batch fermentation for production of PEA

Fed-batch fermentation was carried out at 30°C in a 3 l bioreactor Bioflow 3000 (New Brunswick Scientific, Edison, NJ, USA) which was aerated at 1·5 vvm and stirred at 400 rpm. The initial 1 l medium was inoculated with 40 ml of a culture grown in the same medium but with $(NH_4)_2SO_4$ as the sole nitrogen source. The fermentation medium contained (g l^{-1}): L-phenylalanine – 10, glucose – 20, KH_2PO_4 – 4, $MgSO_4{\cdot}H_2O$ – 0·4, yeast extract – 1. The pH was maintained at 5·0 by 2 mol l^{-1} NaOH and 2 mol l^{-1} H_2SO_4. Glucose levels were tested using a glucose assay kit (Sigma GAGO20) throughout the fermentation and at need glucose was added to maintain a concentration of 15 g l^{-1}.

Analytical methods

Conversion of L-phenylalanine to PEA was determined by HPLC with an Agilent 1100-series instrument (Agilent Technologies, Santa Clara, CA, USA) using an Eclipse XDB C18, 5 μm, 4·6 × 150 mm column (Agilent Technologies). A gradient method comprising water/acetonitrile was applied as follows: 0–6 min 100/0, 10 min 70/30, 16 min 70/30, 20 min 100/0, 22 min 100/0. A diode array detector was used at a fixed wavelength of 215 nm. 1 μl of filtered samples were injected to the column and under these conditions, L-phenylalanine eluted at 4·9 min and PEA at 14·1 min. The concentrations were determined from calibration curves obtained with commercial standards. In order to confirm the production of PEA from L-phenylalanine in the fed-batch culture, a sample of the final broth was analysed by gas chromatography-mass spectrometry (GC-MS) using a GC 6890N (Agilent Technologies) instrument equipped with a capillary column (30 m × 0·32 mm × 0·25 μm), filled with HP-5 ((5%-Phenyl)-methylpolysiloxane) (Agilent Technologies), and an HP-5975 mass spectra detector (Agilent Technologies). The temperature was programmed as follows: T_1 = 80°C, 3 min; dT/dt = 10°C min^{-1}; T_2 = 150°C, split ratio of 1 : 10. Under these conditions PEA eluted at 7·1 min; *m/z* (%): 39 (7·1), 51 (6·5), 63 (6), 65 (18·6), 77 (5·8), 78 (4·2), 91 (100), 92 (56·4), 122 (28·2).

Results

Screening of *S. cerevisiae* strains for the production of PEA from L-phenylalanine

Strain Ye9 is a natural *S. cerevisiae* diploid isolate from Mount Carmel National Park near Haifa, Israel (Katz-Ezov *et al.* 2006). Haploid offspring of this strain obtained by tetrad dissection were previously characterized as thermotolerant, semi-thermotolerant, or nonthermotolerant based on their ability to grow well at 40°C (Nir *et al.* 2008). Thirty-two yeast strains in total were screened for their ability to produce PEA from L-phenylalanine in a

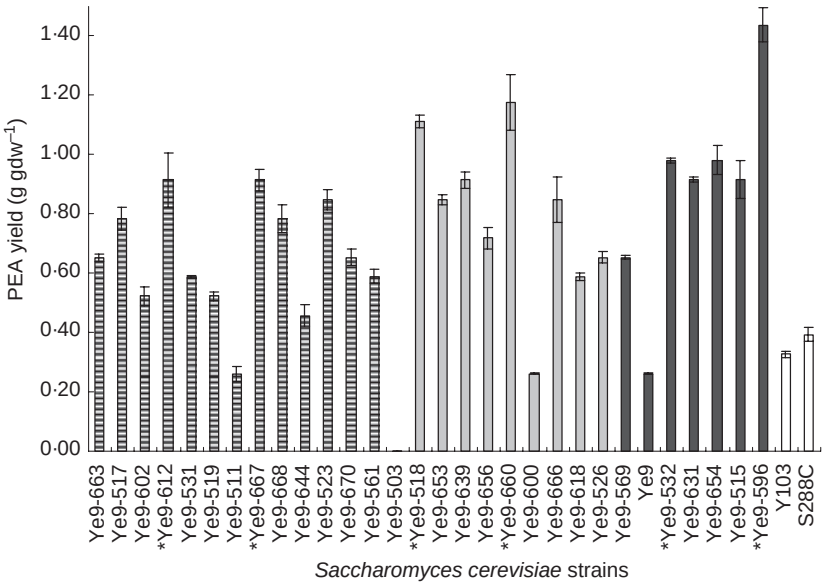

Figure 2 Screening of natural *Saccharomyces cerevisia* strains for production of PEA from L-phenylalanine. Measurements were performed using HPLC after 48 h of growth on minimal media (NE) at 30°C. Thermotolerant strains (▤), Semi-thermotolerant strains (▥), non-thermotolerant strains (■), and laboratory strains (□), are shown. Strains depicted with an asterisk (*) were chosen for further studies based on their high specific yield.

defined medium containing L-phenylalanine as a sole nitrogen source at 30°C (Fig. 2). Each strain was characterized after 48 h of growth by biomass dry weight (gram dry weight per liter – gdw l^{-1}), PEA production (g l^{-1}), and specific yield (PEA concentration per mass). Strains were compared based on their specific yield. PEA production has been previously shown to be growth associated (Wittmann *et al.* 2002; Seo and Ahn 2003) and therefore PEA concentration alone is not a good indication of a strong producer. As the medium composition was not optimized at this stage, a strain could grow relatively slowly but produce high amounts of PEA with respect to its mass. The biomass could be then increased by optimizing the medium. From the results presented in Fig. 2 it is clear that there was no correlation between thermotolerance and PEA production. It was also found that the two laboratory strains Y103 and S288C, and the natural isolate Ye9, had very low production yields (0·10, 0·13 and 0·08 g PEA gdw^{-1}, respectively) in comparison with the offspring of Ye9 (mostly > 0·15 g PEA gdw^{-1}). The two best performing strains from each category (depicted with an asterisk in Fig. 2) were further characterized for growth rate and PEA production at 30°C and 40°C.

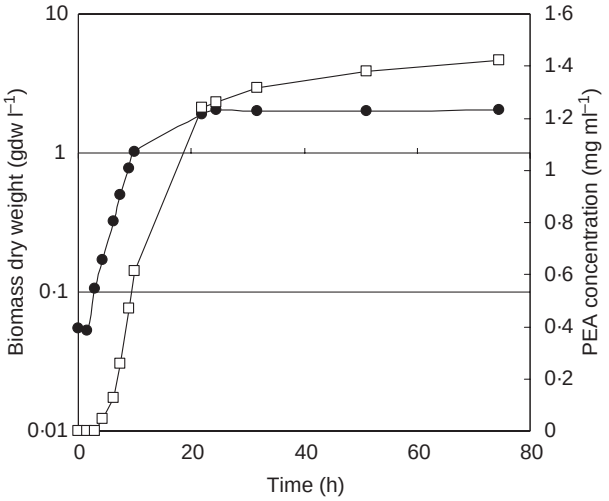

Figure 3 A typical growth and production curve of yeast strains studied in this work. Cells of strain Ye9-612 were grown on minimal media (NE) with an initial OD_{600} of 0·1. Production of PEA was followed by HPLC. Growth rate was determined from the exponential phase in the growth curve (●). Results are an average of two independent measurements with standard deviation less than 10%. (–●–), Growth; (–□–), Production.

Comparison between thermotolerant, semi-thermotolerant and nonthermotolerant strains

Growth and production curves were prepared for each of the six strains, Ye9-612, Ye9-667, Ye9-518, Ye9-660, Ye9-532 and Ye9-596, by measuring biomass dry weight and PEA concentration every 90 min, at 30°C and 40°C. Typical growth and production curves obtained throughout this work are presented in Fig. 3. As expected, PEA production was growth associated. The growth rates (μ), final biomass concentration (gdw l^{-1}), maximum PEA concentrations, and specific yields are summarized in Table 1 and allow a comparison between the strains. It is evident that all growth rates, final biomass dry weight and PEA production decreased at 40°C compared to 30°C, but to a different extent. For the thermotolerant strains (Ye9-612, Ye9-667), there was a decline of only 20% in growth rates

Table 1 Growth rate, final PEA concentration and specific PEA yield of six *S. cerevisiae* strains*

		40°C				30°C			
Strain	Type	$\mu(h^{-1})$	Final biomass (gdw l^{-1})	PEA (g l^{-1})†	Specific yield (g PEA gdw^{-1})	$\mu(h^{-1})$	Final biomass (gdw l^{-1})	PEA (g l^{-1})†	Specific yield (g PEA gdw^{-1})
Ye9-667	T	0·28	0·58	0·10	0·17	0·37	1·80	0·34	0·19
Ye9-612	T	0·31	0·67	0·14	0·21	0·39	1·78	0·42	0·23
Ye9-660	ST	0·26	0·52	0·07	0·13	0·37	1·57	0·46	0·29
Ye9-518	ST	0·23	0·45	0·07	0·16	0·34	1·62	0·37	0·23
Ye9-596	NT	0·07	0·09	NP	NP	0·38	1·99	0·41	0·21
Ye9-532	NT	0·15	0·19	0·02	0·11	0·32	3·05	0·46	0·15

T, thermotolerant; ST, semi-thermotolerant; NT, non-thermotolerant; NP, no product observed.
*Cells were grown at 30°C and 40°C on NE media. Results are an average of two independent measurements with standard deviation lower than 10%.
†PEA concentrations were determined from HPLC injections.

and nearly 70% in maximum biomass dry weight and PEA concentration at 40°C compared to 30°C. For the semi-thermotolerant strains (Ye9-660, Ye9-518), there was a decline of *c.* 30% in growth rates, 75% in final biomass dry weight and 85% in PEA concentration at 40°C compared to 30°C. Amongst the nonthermotolerant strains (Ye9-532, Ye9-596), the decline in growth rate at 40°C was by 55–80%, however the final biomass dry weight was only 5% of its value at 30°C and the PEA concentration was very low. Based on its good performance at both 30°C and 40°C, thermotolerant strain Ye9-612 was chosen for further optimization studies, and nonthermotolerant strain Ye9-596 was chosen for comparison.

Optimization of growth media for higher production yields of PEA

Previous reports (Etschmann *et al.* 2004, 2005) have shown that glucose concentration in the media influences PEA production. Therefore, strain Ye9-612 was grown on minimal media (NE), with different glucose concentrations, ranging from 5 to 40 g l^{-1}, in order to find the optimal glucose concentration for both growth and PEA production. The final dry cell weight concentration, maximum PEA concentration and biomass dry weight yield on glucose are presented in Fig. 4. While the growth rate of strain Ye9-612 was nearly constant at all glucose concentrations (0·5 h^{-1}, results not shown), all of the other parameters were influenced by the glucose concentration. A substantial increase in both biomass dry weight and PEA production was observed at 15 and 20 g glucose l^{-1}, compared with 5 g l^{-1} used in previous experiments (0·85 g PEA l^{-1} and 4·2 gdw l^{-1} at 20 g glucose l^{-1}, compared to 0·42 g PEA l^{-1} and 1·8 gdw l^{-1} at 5 g glucose l^{-1}). Further increase in glucose concentration to 30 and 40 g l^{-1} resulted in a sharp decrease in cell biomass and

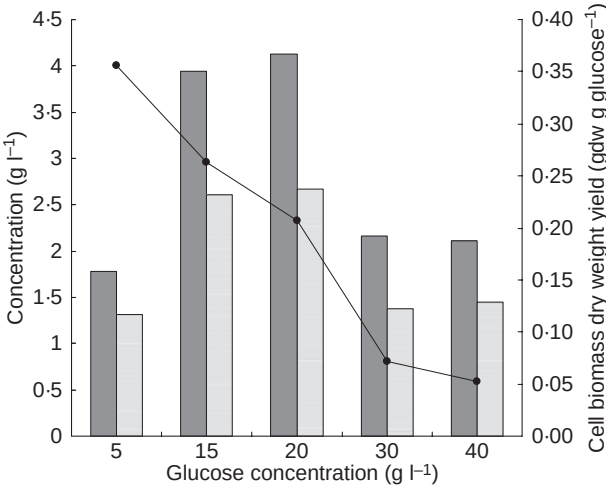

Figure 4 Optimization of glucose concentration for maximum PEA production by strain Ye9-612. Cells were grown on NE medium supplemented with various glucose concentrations at 30°C for 72 h. Production of PEA was followed by HPLC. Results are an average of two independent measurements with standard deviation less than 10%. (■), Biomass dry weight; (▨), PEA concentration; (–●–) Biomass dry weight yield.

PEA concentrations. On the other hand, the biomass dry weight yield (gdw g^{-1} glucose) decreased as the glucose concentration increased, indicating that glucose is used for alcohol production rather than biomass production. As the goal of the research was to maximize PEA production, a concentration of 20 g l^{-1} was further used.

The optimal concentration of L-phenylalanine was evaluated by keeping the glucose concentration at 20 g l^{-1} and varying the amount of the amino acid between 2 and 7 g l^{-1}. A maximum of 0·85 g PEA l^{-1} was obtained at 4 g l^{-1} L-phenylalanine and further increase in

phenylalanine did not result in higher PEA concentrations (results not shown).

Influence of temperature on growth and PEA production of a thermotolerant and a nonthermotolerant strain

Initial results in this research, performed in nonoptimal media (Table 1), indicated that the thermotolerant strains exhibit at 40°C higher growth and production rates than nonthermotolerant strains. In order to examine this trend more thoroughly strains Ye9-612 (thermotolerant) and Ye9-596 (nonthermotolerant) were further evaluated. Both strains were compared in their growth rates, maximum cell density, and PEA production at three different temperatures (30°C, 37°C and 40°C) (Table 2) using the optimal medium developed. As the temperature increased, there was a consistent decline in the growth rate, final biomass dry weight, and PEA production for strain Ye9-596. At 37°C, growth rate decreased by 40%, and PEA concentration decreased by 50% in comparison with the same values at 30°C. At 40°C, growth rate decreased by nearly 70%, and the PEA concentration was negligible. On the other hand, strain Ye9-612 exhibited the same growth rate at 30°C and 40°C, with an unexpected increase at 37°C. The final biomass dry weight decreased gradually with temperature and the final PEA concentration at 40°C was 35% of its value at 30°C, suggesting that Ye9-612 is still viable and productive at a relatively high temperature.

The inhibitory effect of PEA on strains Ye9-612, Ye9-596, and Ye9-532

Stark *et al.* reported that PEA completely inhibits yeast growth at concentrations between 2 and 3 g l^{-1} (Stark *et al.* 2003). We have recently shown that natural *S.cerevisiae* strains exhibiting thermotolerance, are also resistant to osmotic and oxidative stress (Nir *et al.* 2008). It was therefore of interest to examine whether the thermotolerant strain Ye9-612, is also more resistant to high concentrations of PEA in comparison with the nonthermotolerant strains, Ye9-596 and Ye9-532. The three strains were grown in the presence of increasing PEA concentrations, ranging from 0 to 4 g l^{-1}, and both growth rate and maximum biomass dry weight were measured (Fig. 5). Strain Ye9-612 was indeed the most tolerant to PEA by both parameters measured. Strain Ye9-532 was the least tolerant, and the maximum biomass dry weight decreased to a mere 30% of the control value already in the presence of 1 g l^{-1} PEA. Surprisingly, the nonthermotolerant strain 596, had a growth rate profile similar to Ye9-612, however, its biomass dry weight was significantly lower at all PEA concentrations.

Table 2 Growth rates, maximum biomass dry weight, and PEA production by strains Ye9-612 (thermotolerant) and Ye9-596 (nonthermotolerant) at different temperatures*

Strain	Temperature (°C)	$\mu(h^{-1})$	Final biomass dry weight (gdw l^{-1})	PEA (g l^{-1})†
Ye9-612	30	0·35	4·15	0·85
	37	0·46	3·71	0·59
	40	0·36	1·65	0·30
Ye9-596	30	0·44	3·11	0·54
	37	0·27	0·86	0·28
	40	0·14	0·21	0·03

*Cells were grown at 30°C, 37°C and 40°C on NE media containing 20 g l^{-1} glucose for 72 h. Results are an average of two independent measurements with standard deviation lower than 10%.
†PEA concentrations were determined from HPLC injections.

Fed-batch fermentation using strain 612

Strain Ye9-612 was evaluated for its capability to produce high concentrations of PEA in a 3 l fermentor containing 1 l medium. The medium contained initially 20 g l^{-1} glucose and 10 g l^{-1} L-phenylalanine. Cell growth, as well as PEA and glucose concentrations were measured periodically (Fig. 6). Glucose was added batch-wise during the fermentation to maintain a concentration of *c.* 15 g l^{-1}. The final PEA concentration reached 4·5 g l^{-1} after 72 h and to our knowledge, this is the highest concentration reported to date. The final L-phenylalanine concentration remaining in the medium was 4·5 g l^{-1} suggesting a product-per-substrate yield of 0·81 g g^{-1}. The space-time-yield was calculated to be 0·065 g l^{-1} h^{-1}, and the yield of produced PEA per formed biomass was 0·51 g gdw^{-1}.

Discussion

PEA is considered to be one of the most commercially important flavour molecules. The rising consumer's demand in the world's markets for natural food additives, as well as the significant difference in the price of 'natural' and 'chemical' PEA (1000$ compared to 3·5$, respectively) and the costly extraction of PEA from roses or other essential oils, make microbial fermentation a good and efficient option for production of PEA. Such a biotechnological process may allow production of large quantities of PEA in a relatively inexpensive method. Due to the toxic effect of PEA, strains tolerant to high alcohol concentrations are desirable.

In this work, progeny of a natural *S. cerevisiae* isolate from Mount Carmel Park as well as lab strains were evaluated for their ability to produce PEA. The initial product yields (presented in Fig. 2) were similar to results

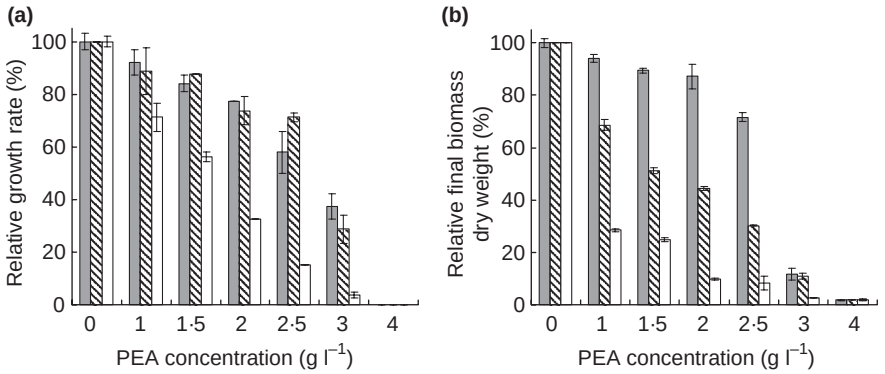

Figure 5 Effect of PEA concentration on growth of selected *Saccharomyces cerevisiae* strains. Cells were grown at 30°C in minimal medium containing $(NH_4)_2SO_4$ as the nitrogen source, and supplemented with various PEA concentrations. Relative growth rate (a) and relative final biomass dry weight (b) are compared to the control without PEA in the medium. Ye9-612 is a thermotolerant strain, Ye9-596 and Ye9-532 are non-thermotolerant strains. (▒), Ye9-612; (▨), Ye9-596; (□), Ye9-532.

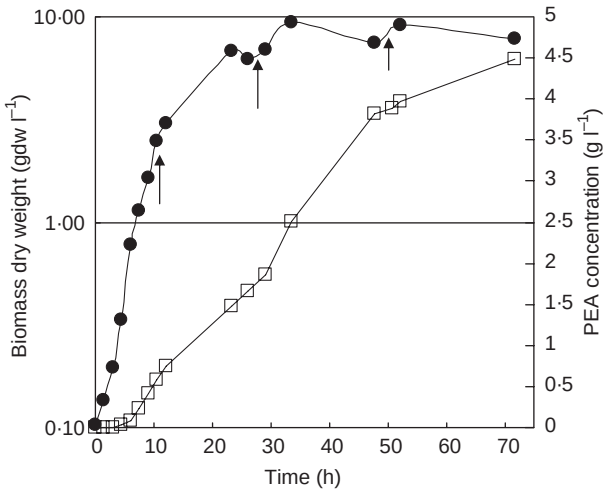

Figure 6 One litre fed-batch fermentation with strain Ye9-612. Cells were grown on NE medium supplemented with 20 g l^{-1} glucose and 10 g l^{-1} L-phenylalanine at 30°C for 72 h. Production of PEA was followed by HPLC. Glucose was added periodically (arrow) to maintain a constant concentration of 15 g l^{-1}. (—●—), Biomass; (—▫—), PEA.

published by other researchers (Fabre *et al.* 1997, 1998; Seo and Ahn 2003), and we found no correlation between PEA production and thermotolerance. Remarkably, the two lab strains (Y103 and S288C) had a very low production yield in comparison with the natural isolates. Their final biomass dry weight was relatively high but their production was quite low resulting in a low specific yield. Among the 30 natural strains screened, only one strain (Ye9-503) did not produce PEA at all under the conditions used. These findings substantiate previous results describing PEA production as dependent on yeast type (Fabre *et al.* 1997; Etschmann *et al.* 2003; Seo and Ahn 2003).

The thermotolerant strains Ye9-612 and Ye9-667 were able to grow and produce substantial amounts of PEA at 40°C (Table 2). As the PEA production is growth associated, the decline in biomass dry weight resulted in a decrease in PEA concentration, however, the specific yield remained quite constant suggesting good adaptation of the cells' metabolism to the stress conditions in opposed to the nontolerant strains. Etschmann *et al.* (2003) evaluated PEA production by various yeast species at elevated temperatures (35–46°C) using ISPR with oleyl alcohol. Only four *Kluyveromyces marxianus* strains produced PEA at temperatures higher than 35°C whereas the two *S. cerevisiae* strains tested were not productive. Results presented here reveal that some *S. cerevisiae* strains are capable of PEA production at elevated temperatures.

Previous works have examined the influence of carbon source concentration in the media on yeast growth and PEA production. Etschmann *et al.* (2003) grew a *K. marxianus* strain in a medium containing sucrose at concentrations ranging from 20 to 60 g l^{-1}. The cell dry weight and ethanol concentrations increased, whereas the PEA concentration remained constant for all sucrose levels. Seo and Ahn (2003) studied *Candida* strains and found constant biomass dry weight and constant PEA levels in the range of 10–50 g glucose l^{-1}. Our results (Fig. 4) differ and show an increase in biomass dry weight and PEA levels up to an optimum at 20 g glucose l^{-1}, followed by a decline and levelling off at 30–40 g l^{-1}. However, the biomass yield on glucose decreased with the increasing glucose concentrations similarly to results reported by Fabre *et al.* (1998) for *K. marxianus* indicating reduced growth at high glucose levels. Under high glucose concentrations, *S. cerevisiae* yeast produce high levels of ethanol *via* the glycolysis pathway. Moreover, in the presence of high concentrations of glucose,

S. cerevisiae can produce ethanol even under aerobic conditions (Crabtree positive yeast). The presence of ethanol in combination with PEA is more toxic than predicted by the summation of the individual effects (Seward *et al.* 1996). Therefore, it is assumed that the growth of yeast cells that were grown on media containing high glucose concentrations (>30 g l^{-1}) was inhibited by the formation of ethanol. The decline in biomass dry weight subsequently caused a reduction in PEA production. Therefore, 20 g l^{-1} was established as the optimal growth and production conditions for *S. cerevisiae* strain Ye9-612. An excess of L-phenylalanine above 4 g l^{-1} did not have a positive effect on growth or PEA production. Similar results were reported by other groups for *S. cerevisiae* GIV2009 (Serp *et al.* 2003), *Candida* sp. S-8 (Seo and Ahn 2003), and *K. marxianus* (Fabre *et al.* 1998), suggesting that L-phenylalanine is not needed in excess to obtain high PEA production.

Strain Ye9-612 was evaluated in the initial screen as thermotolerant due to its ability to grow at 40°C (Fig. 2). Indeed, the increase in temperature from 30°C to 40°C did not affect the growth rate, but did influence the maximum biomass dry weight and subsequently the PEA concentration (Table 2). In contrast, the nonthermotolerant strain Ye9-596 (an offspring of the same parent), showed poor performance in all parameters and could produce only trace amounts of PEA at 40°C. Our recent work on natural *S. cerevisiae* isolates and their progeny, showed that thermotolerance was a good indicator for additional stress tolerant characteristics (Nir *et al.* 2008). For example, a strain showing thermotolerance was also more resistant to oxidative and osmotic stress. This multi-stress resistant phenotype was described by others for yeast, fungi and bacteria (Rahmati-Bahram *et al.* 1995; Cakar *et al.* 2005; Smits and Brul 2005). Extending the multi-stress resistance ability to include tolerance to PEA, was further evaluated in this study. The growth rate of both the thermotolerant (Ye9-612) and the nonthermotolerant strains (Ye9-596, Ye9-532) decreased with increasing PEA concentrations (Fig. 5). The two nonthermotolerant strains differed from one another in their performance, with Ye9-596 being more tolerant than Ye9-532 in the two parameters measured. In the presence of 4 g l^{-1} PEA there was no growth at all as was also shown for *Candida* sp. S-8 (Seo and Ahn 2003). In contrast, the growth rate of *K. marxianus* was already inhibited at 1·2 g l^{-1} PEA (Fabre *et al.* 1998). Biomass dry weight is a more important parameter for obtaining high PEA levels than the growth rate, as seen in Table 2. Despite the fact that the growth rate at 30–40°C remained constant for strain Ye9-612, the PEA concentration decreased due to the lower biomass density, indicating that high biomass is a crucial parameter for obtaining high product concentrations. Strain Ye9-612 displayed 70% of its biomass dry weight compared with the control reaction even at levels of 2·5 g PEA l^{-1}, thus showing outstanding tolerance to the inhibiting product. It can be therefore concluded that strain Ye9-612 has the potential of being an industrial catalyst due to its tolerance to high PEA levels. This high robustness was confirmed in a fed-batch fermentation (Fig. 6) at a one litre scale. The PEA concentration reached 4·5 g l^{-1} after 72 h whereas the highest concentration reported to date was 3·8 g l^{-1} by Stark *et al.* (2003). The space-time-yield was 0·065 g l^{-1} h^{-1} compared with 0·19 g l^{-1} h^{-1} reported by Stark *et al.* however, the initial biomass concentration in the fermentor was only 0·1 gdw l^{-1} compared to 2 gdw l^{-1} in the report by Stark *et al.* (2003), thus the experimental design is different in the two experiments resulting in the differences in space-time-yield. Schmid *et al.* recently reported that for industrial fine chemical production, biotransformations require a minimum space-time yield of 0·1 g l^{-1} h^{-1} and minimum final product concentration of 1 g l^{-1} (Julsing *et al.* 2008). Therefore, the fermentation process will need further optimization to reach the desired space-time-yield. In contrast, the yield of produced PEA per formed biomass was 0·51 g gdw^{-1} (Fig. 6) compared with 0·32 reported for strain Giv 2009 (Stark *et al.* 2003), indicating the high production capability of Ye9-612. The yield of PEA per L-phenylalanine (0·81 g g^{-1}) was slightly above the theoretical yield of 0·74 g g^{-1} (Fabre *et al.* 1998). A yield of 0·8 g g^{-1} was reported previously for *Kluyveromyces polysporus* (Fabre *et al.* 1997). It has been demonstrated that ISPR can facilitate further increase in PEA production (Serp *et al.* 2003; Stark and von Stockar 2003; Etschmann *et al.* 2005; Etschmann and Schrader 2006) and such attempts are currently being pursued in our lab. It is expected that the stress tolerant strain Ye9-612 will be also more tolerant to the presence of an organic ISPR phase and will subsequently enable high production levels at large scale.

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