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Crosses between monokaryons of *Pleurotus sapidus* or *Pleurotus florida* show an improved biotransformation of (+)-valencene to (+)-nootkatone

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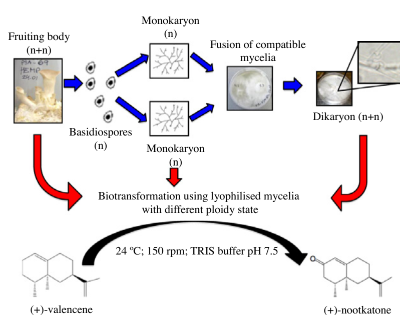
HIGHLIGHTS

- Classical genetics approach improved the biotransformation ability of *Pleurotus*.
- Some of the mono- and new dikaryotic strains gave higher yields of (+)-nootkatone.
- A slow growth rate of monokaryons seemed to correlate with enhanced yield.
- Productive dikaryons were not strictly related to productive parent monokaryons.
- LOX activity of some mono- and new dikaryons was higher than of the parental dikaryon.

GRAPHICAL ABSTRACT



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ABSTRACT

Several hundred monokaryotic and new dikaryotic strains derived thereof were established from (+)-valencene tolerant *Pleurotus* species. When grouped according to their growth rate on agar plates and compared to the parental of *Pleurotus sapidus* 69, the slowly growing monokaryons converted (+)-valencene more efficiently to the grapefruit flavour compound (+)-nootkatone. The fast growing monokaryons and the slow × slow and the fast × fast dikaryotic crosses showed similar or inferior yields. Some slow × fast dikaryons, however, exceeded the biotransformation capability of the parental dikaryon significantly. The activity of the responsible enzyme, lipoxygenase, showed a weak correlation with the yields of (+)-nootkatone indicating that the determination of enzyme activity using the primary substrate linoleic acid may be misleading in predicting the biotransformation efficiency. This exploratory study indicated that a classical genetics approach resulted in altered and partly improved terpene transformation capability (plus 60%) and lipoxygenase activity of the strains.

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1. Introduction

Biotechnological conversions of low-cost agro-industrial by-products, such as terpenes from the distillation of essential plant oils, into more valuable oxygenated derivatives can be achieved using microbial cells or enzymes. These processes outclass traditional chemistry, as they proceed under mild conditions, show better regio- and enantioselectivity, do not generate toxic wastes and result in products labeled 'natural' (Berger, 2009). In particular, the oxidation of (+)-valencene using *Pleurotus sapidus* is attractive to obtain the high added-value compound (+)-nootkatone, an impact flavour of grapefruit with an odor threshold of ~1 $\mu g\ L^{-1}$ water (Fraatz et al., 2009b; Krügener et al., 2010). Recently, the enzyme responsible this transformation was identified as the first

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basidiomycete lipoxygenase, LOX_{Psa} (Plagemann et al., 2012; Zelena et al., 2012).

Numerous studies attempted to introduce fungal genes into expression hosts (Mattanovich et al., 2012; Zelena et al., 2012). Cytochrome P450 enzymes from bacteria and fungi were intensively studied and expressed in *Escherichia coli* and in more food compatible hosts, such as *Saccharomyces cerevisiae*, to convert (+)-valencene into (+)-nootkatone. Because of the overall complexity and instability of this class of enzymes, the need for cofactors and accumulation of the product in lipophilic cell compartments, the product concentrations secreted remained in the microgram or low milligram range per liter. To make it worse, the second oxidation step from the alcohol to the often more odor-active carbonyl is not catalyzed by CPY450s (Fraatz et al., 2009a; Gavira et al., 2013; Girhard et al., 2009). Furthermore, recombinant microorganisms are refused by the European societies, and, based on current legislation (European Directive 2001/18/EC), strains produced along genetic transformation or mutagenic treatments may not be suitable for "natural or safe processes" (Gounaris, 2010). Therefore, classical genetic approaches represent an option to improve the product yields of fungal biotechnology. These are based on the mating of two monokaryotic compatible strains, whose hyphae are able to fuse and give rise to a dikaryotic mycelium in which the two parental nuclei (haploid) are maintained indefinitely in paired association during vegetative growth (Kaur et al., 2008; Kothe, 2001).

The use of edible fungi in biotechnology is novel and luring, because they are food and possess a long history of safe use. Their products could thus be directly introduced in food, pharmaceutical and cosmetic applications. *Pleurotus* species offer a number of additional technical advantages, i.e. sequenced genome, short life cycle, easy cultivation in solid and liquid media, and first biotechnological applications have become known (Cohen et al., 2002; Krings and Berger, 2010). In *Pleurotus* species, the mating system is controlled by two unlinked *mat* loci, *A* and *B* (bifactorial). For a successful mating between monokaryons, *A* and *B* have to be of different allelic specificity (*x* and *y*). This system is referred to as tetrapolar, since it generates four different incompatibility types in the monokaryotic offspring of a dikaryon: *AxBy*; *AyBx*; *AxBx*; *AyBy*. Basidiospore germination produces a mycelium in which all cells are monokaryotic (n). Two monokaryotic mycelia belonging to different mating types can fuse to form a dikaryotic mycelium ($n + n$), until a set of environmental conditions triggers fruiting body formation. Karyogamy occurs within the basidia, immediately followed by meiosis producing four uninucleate spores. The pure lines derived by mating show different traits, because each of the monokaryon is a genetic variant resulting from meiosis. Subsequent crosses of basidiospore-derived monokaryons combines their features into a new dikaryon. The new dikaryotic strains may carry improved desirable traits, such as mycelium growth rate, higher enzyme production, variability on enzyme isoforms expression, and better fruiting efficiency (del Vecchio et al., 2012; Eichlerova and Homolka, 1999; Kaur et al., 2008; Lomascolo et al., 2003).

In the present work, three strains of *Pleurotus* were used to create mono- and new dikaryotic strains by classical genetics techniques and screened for their capability to transform (+)-valencene. Using a well-investigated lipoxygenase as the test trait, it was hypothesized that the random events of meiosis would result in broadened metabolic diversity, and that some higher yielding mono- and dikaryons would emerge. It was also of interest, if new dikaryotic crosses fused from high yielding monokaryons showed an even better biotransformation activity. Such biotransformations using low-cost industrial by-products as bioresources are a green, bioeconomic strategy to produce value-added natural products.

2. Methods

2.1. Microorganisms

The dikaryotic strains *P. sapidus* 8266 (PSA-69), *P. sapidus* 195.92 (PSA-224) and *Pleurotus florida* 216 (PFL-216), obtained from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH/DSMZ), Braunschweig, Germany, representated a low, an average and the best nootkatone producing strain from a large set of *Pleurotus* cultures pre-screened. The strains were cultivated on *Petri* dishes containing glucose/asparagine/yeast extract medium (SNL) as described (Onken and Berger, 1999) with 2% agar and incubated at 25 °C for 7 days. Stock cultures of strains were maintained in the same medium and stored at 4 °C.

2.2. Culture conditions for fructification and isolation of basidiospores

Dikaryotic strains of *Pleurotus* (PSA-69, PSA-224 and PFL-216) were cultivated in 500 ml flasks containing 10 g of hemp with a water content of 70% (Omarini et al., 2009). The flasks were autoclaved at 121 °C for 1 h. Each flask was inoculated with four agar plugs (10 mm diameter) of mycelia (7-day old culture) grown on SNL-agar *Petri* dish, and left at 25 °C in the dark until the mycelia completely covered the substrate. Fructification was promoted by opening the flask, and placing them in the presence of daylight in a chamber at 15 ± 5 °C and 90% relative humidity. Once the fruiting bodies matured (after 6–8 days), they were harvested, and individual basidiocarp derived from each strain was used to obtain spore prints (Eichlerova and Homolka, 1999; Kaur et al., 2008). Basidiospores were collected from the fruiting bodies in the following way: the pileus was cut off with a scalpel under sterile conditions and transferred into a *Petri* dish onto a sterile piece of silver paper (Eichlerova and Homolka, 1999). When this paper was covered with the spores, the pileus was removed and spore suspensions were prepared in 1 ml sterile physiological salt solution (0.9% NaCl) and stored at 4 °C. The basidiospores were plated in SNL solid medium in *Petri* dishes after appropriate dilution, spread across the surface of the hardened agar using a sterile cell spreader to obtain distinct monosporic colonies and incubated at 25 °C. Colonies formed were transferred onto SNL agar *Petri* dishes. Each colony was examined by phase-contrast microscopy for clamp connections (specialized structures which allow nuclei distribution into daughter cells) to detect the appearance of colony characteristics specific for dikaryons (Kothe, 2001). 100 colonies lacking clamp connections were sub-cultured in SNL agar *Petri* dishes, incubated at 25 °C for 7 days and then stored at 4 °C.

2.3. Mating test

Mating type of the single spore isolate of each *Pleurotus* strain was analyzed using the two-point inoculation technique. Briefly, small pieces of mycelium of two monokaryons (agar plugs, 5 mm^2 diameter, cork borer) were inoculated close to each other in *Petri* dishes containing SNL agar medium. After 5–7 days of growth they formed a large contact zone. A piece of mycelium was cut off from the contact zone and examined under the microscope for the presence of true clamp connections (dikaryons), unfused (false) clamp connections (common-*B* heterokaryons), or absence of clamp connections (common-*A* heterokaryons) (Kothe, 2001). In order to isolate testers representing the four incompatibility types present in each *Pleurotus* strain used in this work (PSA-69, PSA-224 and PFL-216) ten monokaryons derived from each strain were randomly selected and intercrossed to identify the four expected classes (*AxBx*, *AxBy*, *AyBx*, *AyBy*) for this tetrapolar fungus species according to the expected phenotypes for the

common-*A* and common-*B* heterokaryons (Kothe, 2001). The four testers obtained for each *Pleurotus* strain were used to determine the incompatibility types of the remaining members of the offspring. Subsequent crosses of monokaryons were determined through sorting by mating types.

2.4. Pre-screening experiments on (+)-valencene-SNL agar plates: dikaryotic strains

The ability of each dikaryotic parental strain to grow on solid media with different terpene concentrations was evaluated and expressed as growth rate (mm/d) to select the best conditions for continuing with the monokaryons selection (Bicas and Pastore, 2007; Uenojo and Pastore, 2010). The three *Pleurotus* strains were used to inoculate *Petri* dishes containing SNL-agar medium with a different concentration of (+)-valencene (0.0%, 0.5%, 1.0%, 2.0% and 2.5%, v/v) and pure SNL-agar medium as the control. A 5 mm^2 piece of actively growing mycelium of each strain was placed in the center of a *Petri* dish and incubated at 25 °C in the dark. The linear growth rate was measured as the radial increase of the colony per day. Four orthogonal measures were made per plate every 2 days. Strains with a satisfactory growth were considered resistant to that concentration of terpene (v/v). All tests were carried out as independent triplicates.

2.5. Monokaryons screening and dikaryon formation

Screening experiments were performed to select the most valencene tolerant monokaryons (Mks). One hundred monokaryons derived from each *Pleurotus* strain were cultivated in *Petri* dishes containing SNL medium plus the effective dose of (+)-valencene (SNL-Val) and in SNL-agar medium. All experiments were carried out under the same experimental conditions as used for the parental dikaryotic strains. The colony growth was measured every 2 days as above. Ten monokaryotic (Mk) strains, five with a higher and five with a slower growth rate were selected to obtain new dikaryotic (Dk) strains and to carry out the (+)-valencene transformation.

New dikaryons (Dks) were obtained by using the two-point inoculation technique. The mating was done by crossing two fast and compatible Mks (F × F), two slow Mks (S × S) or one fast and one slow Mk (F × S).

2.6. Culture conditions for transformation experiments

Selected strains were inoculated into a glucose/asparagine/yeast extract liquid medium (Onken and Berger, 1999) and grown aerobically at 24 °C and 150 rpm in an orbital shaker (Multitron, Infors, Bottmingen, Switzerland). Experimental cultures (300 ml shake flask, 200 ml medium volume) were inoculated with 20 ml five-days-old precultures that were grown on the same medium and homogenized using an ultraturrax (Janke & Kunkel, Staufen, Germany) prior to inoculation. After 6 days of growth, the culture was harvested, separated from the supernatant by centrifugation, and the fungal mycelium was washed twice with distilled water. The obtained biomass was lyophilised, using a VaCo 2 (Zirbus Technology, Bad Grund, Germany), and stored at −20 °C until usage (Fraatz et al., 2009b).

2.7. Transformation of (+)-valencene with lyophilisates of Pleurotus strains

The transformation of (+)-valencene was carried out in glass vials (4 ml) in horizontal position in an orbital shaker (150 rpm, 25 mm shaking diameter; Multitron, Infors) at 24 °C for 16 h in the absence of light (Fraatz et al., 2009b; Krügener et al., 2010). 1.4 µl (+)-valencene (Fluka, ⩾90%) and 1.5 ml TRIS–HCl buffer (20 mM, pH 7.5) were added to 50 mg *Pleurotus* lyophilisate (mono- or dikaryons). A biological blank was performed in the same way but with mycelium deactivated for 30 min at 95 °C before addition of (+)-valencene. A chemical blank was performed in the same way, but without mycelium, to estimate the extent of chemical transformation. After 16 h of bioconversion the solution was extracted with 1 ml n-hexane after adding thymol (1 g/l) dissolved in n-hexane as internal standard. The upper phase was withdrawn, dried (Na_2SO_4) and analyzed by capillary GC–FID and mass spectrometry (HRGC–MS).

2.8. Identification and quantification of transformation products

Samples were analyzed using a Perkin Elmer Autosystem XL (USA), equipped with an autosampler, S/S inlet (1:10), FID detector and a MN Optima Wax capillary column (30 m × 0.32 mm i.d., 0.25 µm film thickness; Macherey-Nagel, GmbH & Co, Germany) and a flame ionization detector (250 °C, 40 ml min^{-1} H_2, 450 ml min^{-1} air flow, 45 ml min^{-1} make up gas) using the following temperature program: 70 °C (hold for 1 min) to 230 °C with a rate of 10 °C min^{-1} and hold for 5 min. Hydrogen was used as carrier gas (58.4 kPa head pressure, 2.0 ml min^{-1} constant flow).

High resolution gas chromatography mass spectrometry (HRGC–MS) analysis was carried out using a Fisons GC 8000 gas chromatograph and a Fisons MD 800 mass selective detector (interface: 230 °C, ion source: 200 °C, quadrupole: 100 °C, electron impact ionization (ei, 70 eV), scan range m/z 33–300 amu) as described (Krügener et al., 2010). Analytical conditions were: 30 m × 0.32 mm i.d. × 0.25 µm DB-WAX (J & W scientific, Folsom, USA) with 1 ml min^{-1} helium carrier gas using a temperature program: 70 °C (hold for 1 min) with a rate of 10 °C min^{-1} to 230 °C hold for 5 min.

Identification of biotransformation products was achieved according to linear retention indices and mass spectrum compared to a digital library (Wiley 08/NIST 08).

2.9. LOX_{Psa} activity

Enzyme activity was determined spectrophotometrically by monitoring the increase in the absorbance at 234 nm due to the transformation of linoleic acid to the respective conjugated hydroperoxydiene (Plagemann et al., 2012). The assay was carried out in an UV-transparent 96-well microtiter plate containing 5 µl enzyme solution (supernatant obtained by incubation of 1 g of lyophilised biomass in 100 ml TRIS–HCl buffer (pH 7.5) during 4 h at 4 °C by shaking) and 175 µl TRIS–HCl buffer (pH 7.5). The reaction was initiated by adding 40 µl of a freshly prepared 2 mM substrate solution which was made up of 20 µl (18 mg) linoleic acid, 30 µl (33 mg) Tween 20, and 60 µl 1 M NaOH in a 2 ml volumetric flask and filled up with distilled water. This mixture was diluted with distilled water to the appropriate final concentration of 2 mM linoleic acid. Absorbance at 234 nm was recorded every 30 s and monitored for 20 min using a microplate reader (Synergy 2, Biotek, Bad Friedrichshall, Germany) which was kept at 30 °C.

Enzyme activity was calculated on the basis of the molar extinction coefficient of the conjugated diene hydroperoxides at 234 nm ($\varepsilon = 2.5 \times 10^4\ M^{-1}\ cm^{-1}$). Blanks were carried out using 40 µl buffer instead of enzyme solution. All experiments were performed in triplicate.

2.10. Statistical analysis

SPSS for Windows V8.0.1S software (SPSS Inc., Chicago, IL) was used to perform the normality tests (Kolmogorov–Smirnov) and

analyses of variance (ANOVA and Tukey's Post-hoc test). The correlation was established by using Pearson's correlation coefficient.

3. Results and discussion

3.1. Production of Pleurotus monokaryotic strains

The two edible fungi, *P. florida* strain PFL-216 and *P. sapidus* strains PSA-69 and PSA-224, differ in several morphological and physiological features and have been extensively characterized for their ability to produce enzymes of industrial interest, such as laccase (del Vecchio et al., 2012; Linke et al., 2005), lipoxygenase (Krügener et al., 2010; Zelena et al., 2012), and esterases (Linke et al., 2009), and for their capability to transform terpene compounds (Krings and Berger, 2010). In order to produce new dikaryotic strains with increased capabilities in terpene transformation, basidiospore derived monokaryons obtained from these *Pleurotus* strains were isolated, screened and analyzed.

As a preliminary step, the strains were cultivated in flasks containing hemp as a substrate and incubated for 20 days at 25 °C. Afterwards, the environmental conditions were changed to promote the fructification and one basidiocarp of each strain was harvested and used to obtain the spore prints. The spores were collected and plated in SNL solid medium. Microcolony progeny were analyzed microscopically, and the monokaryotic state was confirmed by the absence of mycelial clamp connections (Eichlerova and Homolka, 1999; Kothe, 2001). One hundred monokaryons (Mks) for each parental *Pleurotus* strains were isolated in order to obtain a representative number of offspring with desirable traits to be screened and to produce new dikaryotic strains. The monokaryotic strains were kept at 4 °C and used in the following experiments.

3.2. Pre-screening experiments: analysis of mycelium growth rates of parental Pleurotus species

In fungi, the growth rate is a polygenic and quantitative trait frequently used to estimate the ability of a strain to colonize a substrate, spread a new niche or exploit a new environment (Haritash and Kaushik, 2009; Uenojo and Pastore, 2010; van der Nest et al., 2009). In addition, this trait is easy to measure and has been widely used in screening and selection assays among strains with different ploidy states (Bicas and Pastore, 2007; Clark and Anderson, 2004; Eichlerova and Homolka, 1999; Hiscox et al., 2010; Rottava et al., 2010).

A pre-screening was carried out to select the best (+)-valencene concentration for the subsequent screenings with the monokaryotic strains (Fig. 1). Most microorganisms are unable to thrive at elevated concentrations (⩾2%) of terpene hydrocarbons because of the cytotoxicity of these unpolar compounds; 2.5% (v/v) was the highest concentration used (Bicas and Pastore, 2007; Onken and Berger, 1999). The growth of three *Pleurotus* species (PSA-69, PSA-224 and PFL-216) was monitored during the time of cultivation (mm/d). The growth rate achieved was significantly affected ($p < 0.05$) by the presence of valencene in the culture media (from 0.5% to 2.5%). All of the strains were able to grow in the presence of elevated concentration of (+)-valencene but grew more slowly than those in the control SNL medium. Only slight differences in the growth rate were found among strains growing at different concentrations of the terpene (Fig. 1). It was concluded that the presence of 1% of this lipophilic compound was sufficient to present the desired metabolic challenge to these strains; thus it was selected to carry out the screenings with the monokaryons.

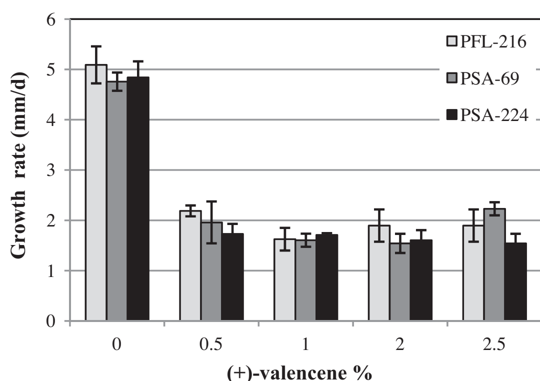

Fig. 1. Growth rate (mm/d) of the three *Pleurotus* strains on *Petri* dishes containing SNL solid medium with different concentrations of (+)-valencene (0.0–2.5%, v/v).

3.3. Monokaryons screening experiments

The screening experiment performed with one hundred monokaryons (Mks) derived from each of the *Pleurotus* strains was conducted to select tolerant Mk strains derived from each parental dikaryon (Dk) with an expected ability to transform (+)-valencene. Growth rate was used as a general fitness parameter to evaluate the capability of the offspring to grow on a selective SNL medium supplemented with (+)-valencene (1%, v/v) and on SNL, as control medium.

Mycelial growth of Mks derived from the three *Pleurotus* strains displayed a continuous distribution in both culture media (Fig. 2) and represented a quantitative trait, consistent with results obtained with other fungi (Olson, 2006; van der Nest et al., 2009). In all cases, the growth rate achieved on SNL-Val was lower than on SNL medium due to the presence of the inhibitory terpene. Most of the monokaryons showed a medium growth rate, but the wide phenotypic variation among the different isolates confirmed that the growth rate is a complex, variable genetic trait.

Mks derived from PSA-69 ranged from 1.59 to 4.67 mm/d on SNL (10% of the isolates were as fast as the parental Dk) and 1.35–3.01 mm/d on SNL-Val (46% of the isolates were at least 0.5-fold faster than the parental Dk); PSA-224 Mks ranged from 1.80 to 4.67 mm/d on SNL (3% of the isolates were similar to the parental PSA-224) and 1.17–3.05 mm/d on SNL-Val (12% of the isolates were at least 0.5-fold faster than the parental Dk); Mks from PFL-216 ranged from 2.59 to 4.91 mm/d on SNL (all the isolates were slower than the parental Dk) and 1.52–3.03 mm/d on SNL-Val (34% of the isolates were at least 0.5-fold faster than the parental Dk). The results showed that (i) a few isolates from PSA-69 and PSA-224 grown on SNL medium reached a similar growth rate as the corresponding parental Dk, and (ii) most of the Mks grown on SNL-Val showed a similar or higher growth rate than their corresponding parental Dk, proving that it was possible to obtain better adapted phenotypes able to colonize a selective medium faster. The good performance obtained by each set of Mks on SNL-Val can be explained by the great flexibility of metabolic processes and enzymes involved enabling a saprobic cell to feed on complex organic molecules in variable environments (Rottava et al., 2010). This fact highlighted the need to compare the development of fungi in different environments, because fungal strains react differently to a stimulus by the medium composition. However, as noted by Bicas and Pastore (2007) and Rottava et al. (2010), the degradation of a recalcitrant substrate does not necessarily guarantee, but frequently indicate a high transformation activity.

The growth profile obtained by each set of MKs growing on SNL-Val medium eventually allowed to select representative Mks, five with high and five with slow growth rates to generate the

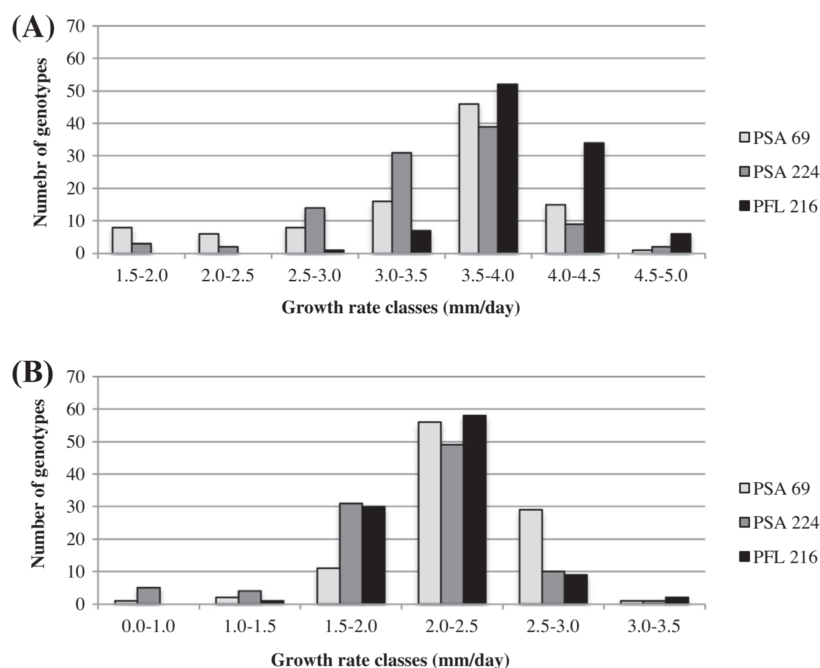

Fig. 2. Frequency distribution of vegetative growth rate (mm/d) of the monokaryons derived from the three *Pleurotus* strains grown on (A) SNL and, (B) SNL-Val (1%, v/v) agar plates.

new Dk strains and to evaluate their potential transformation capability.

3.4. (+)-Valencene transformation using lyophilised mycelia of selected monokaryons and new dikaryons

Little data is available on quantitative metabolic differences of basidiomycete cell lines with different ploidy states. Monokaryotic cells are an alternative to genetically modified organisms and provide a simpler genetic system than dikaryons. Mks were stable during long-term cultivation, an important condition for industrial applications (Clark and Anderson, 2004). In order to explore the (+)-valencene transformation capability, first the selected representative set of Mks of each *Pleurotus* strains were evaluated and compared with the corresponding parental strains. In addition, compatible Mks of each strain were chosen to generate new Dks strains to be used for the transformation reaction.

Fig. 3 A, shows the yields of (+)-nootkatone achieved by all the strains after 16 h of biotransformation. Results showed (i) a wide phenotypic variation in terms of (+)-nootkatone yields among the three *Pleurotus* strains and among the progeny derived from each one indicating a significant genetic variability among the isolates (Fig. 3 A); (ii) that the parental Dk PSA-69 and Mks selected thereof attained higher (+)-nootkatone yields reaching 10 to 30-fold concentrations compared to average values obtained by Dk-PSA 224, Dk-PFL 216 and their corresponding Mks. The yields were similar to those reported by Fraatz et al. (2009b) and higher than those using the purified recombinant enzyme ValOx (=LOX_{Psa}, Zelena et al., 2012); (iii) the transformation efficiency was higher for the slow Mks derived from PSA-69 compared with the faster ones; (iv) growth rate and conversion ability among the Mks of PSA-69 was negatively correlated ($r^2 = -0.63$; $p < 0.05$). It is conceivable that slower adaptation to a selective medium may have promoted physiological changes to survive in an unfavorable environment by developing mechanisms counteracting the presence of a basically cytotoxic terpene hydrocarbon. In clear contrast, the higher growth rate achieved by some strains was not linked with a high transformation yield. An accelerated primary metabolism will not result in a faster formation of the LOX_{Psa}, if the enzyme's functional role is more related to secondary metabolic processes.

The transformation profiles of the Mks strains offered a starting point to study the effects of the monokaryotic and dikaryotic condition on transformation capability. New dikaryotic strains of each *Pleurotus* strains were generated by mating compatible Mks derived of each one (S × S, F × F and S × F) in order to increase the phenotypic diversity (Fig. 3 A).

The (+)-nootkatone yields achieved by the new Dks after 16 h of transformation showed again a wide phenotypic diversity. The new dikaryons derived from the crossing of PSA-69 Mks were four to twelve-folds higher than the average values obtained by the new Dks of PSA-224 or PFL-216. In particular, five new Dks derived from PSA-69 (31 × 74, 21 × 8, 3 × 88, 4 × 21, and 37 × 66) showed significantly higher yields of (+)-nootkatone than the parental Dk ($p < 0.05$). In four out of the five top performers, one nucleus originated from a slowly growing Mk. Thus, the classical genetics approach resulted in improved transformation capabilities. This was most noticeable in the new Dks derived from PSA-69, which produced 20–60% more (+)-nootkatone compared with the parental Dk. The same trend was observed for the new Dks derived from PSA-224 and PFL-216, but on a lower level.

The performance of the new strains derived from PSA-69 could not be strictly related to the performance of each component Mk: High producing Dks did not always result from the fusion of high producing Mks. This suggested that yet unknown factors are involved in the over-all performance of cells with coexisting nuclei. Different interactions of genes (e.g. overdominant, dominant, epistatic, additive) between the two non-fused nuclei could be the main source of phenotypic variance of parameters, such as growth rate, enzyme production, gene expression, and synthesis of metabolites (Chen et al., 2013; Clark and Anderson, 2004; Gay and Debatud, 1987; Hiscox et al., 2010). In addition, dikaryons may undergo somatic recombination of genomes during vegetative growth allowing the shuffling of genes between nuclei without complete reassortment as in the course of meiosis (Anderson and

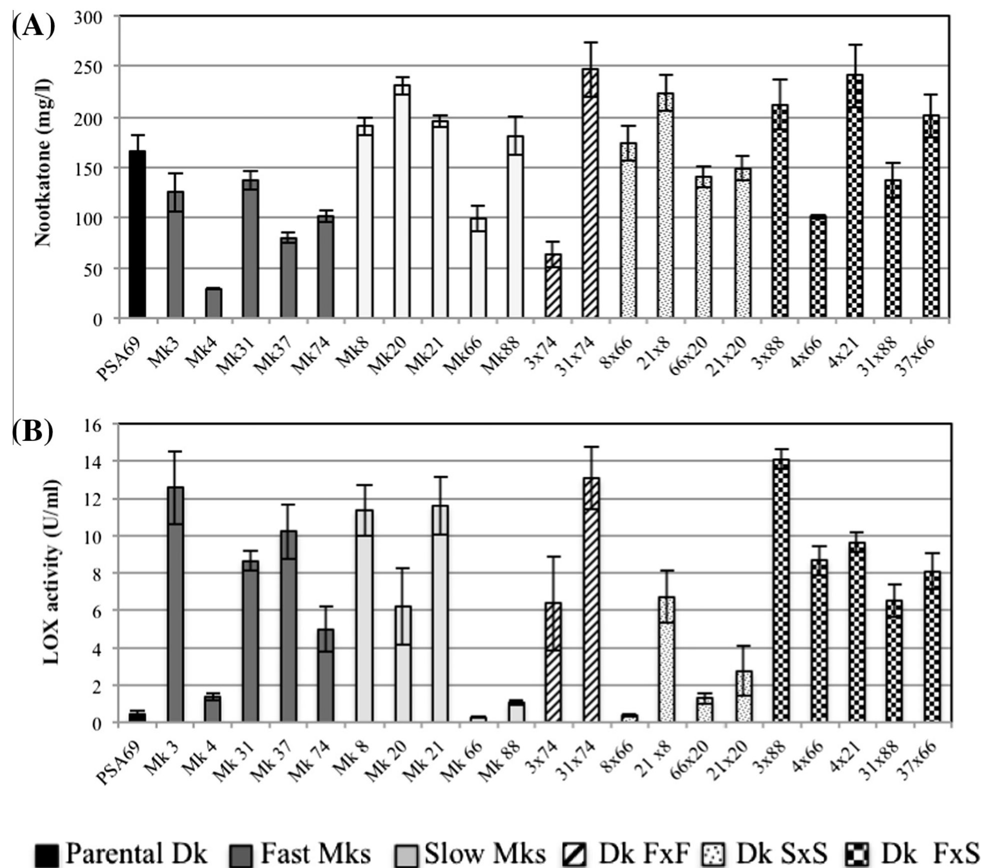

Fig. 3. (A) (+)-Nootkatone (mg/l) and (B) LOX_{Psa} (U/ml) production by the parental dikaryon PSA-69, selected monokaryons and new dikaryons obtained by classical genetics techniques.

Kohn, 2007). Additional genetic variation in Dks could result from the distance between the two nuclei within a cell, as reported for *Schizophyllum commune* (Schuurs et al., 1998). Due to this variation, dikaryotic strains were expected to have increased adaptive potential (in terms of growth rate) relative to monokaryons during long-term propagation (Anderson and Kohn, 2007; Clark and Anderson, 2004).

Previous work indicated that slowly growing new Dks gave higher enzyme yields (Eichlerova and Homolka, 1999). This is in line with the present findings that some of the best producing strains contained at least one monokaryotic component with a slow growth rate. The modest performance of all slow × slow crosses and the different performance of the two fast × fast crosses show however, that the growth rate is not reliable for assessing a single metabolic trait. Markers to predict the outcome of a fusion experiment are not available, and every new Dk must be examined for its desired metabolic efficiency. As a side note, the metabolic diversity of strains (Mks and Dks) even from the same fruiting body may explain the common observation of fungal laboratories that results obtained with a particular dikaryotic strain are often difficult to reproduce quantitatively.

3.5. LOX_{Psa} activity evaluation

It was established by previous work that the transformation of (+)-valencene by lyophilised mycelia of *P. sapidus* was catalyzed by LOX_{Psa}, a lipoxygenase (Krügener et al., 2010; Plagemann et al., 2012; Zelena et al., 2012). As was noted earlier (del Vecchio et al., 2012; Eichlerova and Homolka, 1999), mycelial growth rate and enzyme production levels are not generally correlated. The present data show a low correlation ($r^2 = 0.22$, $p < 0.05$) between growth rate and LOX_{Psa} production for the Mks derived from PSA-69.

The LOX_{Psa} activity produced by selected strains with different ploidy state derived from PSA-69 was generally higher than in the parental dikaryotic strain (Fig. 3 B). Similar improvements were obtained for other enzymes in *P. ostreatus* and *Hebeloma* species (del Vecchio et al., 2012; Gay and Debatud, 1987). In agreement with findings for *P. ostreatus* (Eichlerova and Homolka, 1999), many of the high LOX and (+)-nootkatone producing new Dks contained one nucleus originating from a slowly growing Mk.

The classical genetics approach represented an alternative to improve a desired trait of a fungus. The Mk 3, Mk 37, Mk 8, Mk 21 and the new Dks 31 × 74 and 3 × 88 proved to be the best LOX_{Psa} producer strains ($p < 0.05$), reaching more than 10.0 U/ml. The high variability of enzyme activity among mono- and dikaryotic strains agrees with results of other authors (del Vecchio et al., 2012; Eichlerova and Homolka, 1999; Gay and Debatud, 1987; Hiscox et al., 2010; Tello et al., 2001). The wide range of enzyme activities produced by Mks and Dks indicated that the system controlling LOX_{Psa} activity may be polygenic. The variability showed by monokaryons can arise from the segregation of alleles during meiosis (i.e. after genetic recombinations which could affect regulator and/or promoter genes of specific enzymes) or could indicate heterozygosity of genes responsible for the synthesis and secretion of specific enzymes (Eichlerova and Homolka, 1999; Lomascolo et al., 2003). The variation of LOX activity in dikaryotic strains involves additive (e.g. 31 × 74; 3 × 88) and non-additive (e.g. overdominance in 4 × 66) effects as reported by Gay and Debatud (1987) and Castanera et al. (2013). Furthermore, a number of

new Dks with significantly better performance than the parental PSA-69 often showed lower enzyme activity than one of their component Mk and similar activity to its other component (e.g. 3×74, 8×66). This result showed that the variation among dikaryons could not be strictly related to the variations of the parental monokaryons, thus indicating that the genetic control of LOX activity probably involved a non-additive effect. Evaluating different ligninolytic enzymes produced by *Trametes versicolor* a partial dominance of one component Mk in the enzyme production of artificial dikaryons was reported (Hiscox et al., 2010).

When the activity of LOX_{Psa} of Mks and the new Dks derived from PSA-69 was compared with the yields of (+)-nootkatone, a low correlation was obtained ($r^2 = 0.30$; $p < 0.05$). This could be attributed to a mutated sequence of the LOX_{Psa} gene or the presence of other not yet identified LOX isoenzymes not directly implicated in the catalytic reaction. A more likely explanation may be derived if a co-oxidation type of the reaction is supposed: While the transient occurrence of tertiary hydroperoxides of (+)-valencene in the course of the reaction was unequivocally demonstrated (Krügener et al., 2010), the natural abundance of linoleic acid as the primary substrate of the LOX_{Psa} and the concentration of radical scavenging or promoting compounds (anti- or prooxidants) in the catalytic lyophilisates may be subject to biological deviations.

4. Conclusions

This exploratory study showed that a classical genetics approach created *Pleurotus* strains with a wide range of lipoxygenase activity, as measured directly and as indicated by the transformation of (+)-valencene to (+)-nootkatone. Selected mono- and dikaryons may catalyze other terpene transformations for the production of optically pure compounds essential for the food, fragrance and pharmaceutical industries. Factors increasing the genetic plasticity of basidiomycetes improve the chances to encounter over-producers, but prevent a more targeted approach. The outcome of experiments evaluating the long-term stability of the best Dks and the component Mks will decide on future applications in fungal biotechnology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.08.061.

References

Anderson, J.B., Kohn, L.M., 2007. Dikaryons, diploids, and evolution. In: Heitman, J., Kronstad, J.W., Taylor, J.W., Casselton, L.A. (Eds.), Sex in Fungi: Molecular Determination and Evolutionary Implications. ASM Press, Washington, D.C., p. 542.

Berger, R.G., 2009. Biotechnology of flavours – the next generation. Biotechnol. Lett. 31, 1651–1659.

Bicas, J.L., Pastore, G.M., 2007. Isolation and screening of D-limonene-resistant microorganisms. Braz. J. Microbiol. 38, 563–567.

Castanera, R., Omarini, A., Santoyo, F., Perez, G., Pisabarro, A.G., Ramirez, L., 2013. Non-additive transcriptional profiles underlie dikaryotic superiority in *Pleurotus ostreatus* laccase activity. PLoS ONE 8, e73282.

Chen, B., Gui, F., Xie, B., Deng, Y., Sun, X., Lin, M., Tao, Y., Li, S., 2013. Composition and expression of genes encoding carbohydrate-active enzymes in the straw-degrading mushroom *Volvariella volvacea*. PLoS ONE 8, e58780.

Clark, T.A., Anderson, J.B., 2004. Dikaryons of the basidiomycete fungus *Schizophyllum commune*: evolution in long-term culture. Genetics 167, 1663–1675.

Cohen, R., Persky, L., Hadar, Y., 2002. Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*. Appl. Microbiol. Biotechnol. 58, 582–594.

del Vecchio, C., Lettera, V., Pezzella, C., Piscitelli, A., Leo, G., Birolo, L., Sannia, G., 2012. Classical breeding in *Pleurotus ostreatus*: a natural approach for laccase production improvement. Biocatal. Biotransform. 30, 78–85.

Eichlerova, I., Homolka, L., 1999. Preparation and crossing of basidiospore-derived monokaryons – a useful tool for obtaining laccase and other ligninolytic enzyme higher-producing dikaryotic strains of *Pleurotus ostreatus*. Anton. Leeuw. Int. J. G. 75, 321–327.

Fraatz, M.A., Berger, R.G., Zorn, H., 2009a. Nootkatone – a biotechnological challenge. Appl. Microbiol. Biotechnol. 83, 35–41.

Fraatz, M.A., Riemer, S.J.L., Stober, R., Kaspera, R., Nimtz, M., Berger, R.G., Zorn, H., 2009b. A novel oxygenase from *Pleurotus sapidus* transforms valencene to nootkatone. J. Mol. Catal. B Enzym. 61, 202–207.

Gavira, C., Hofer, R., Lesot, A., Lambert, F., Zucca, J., Werck-Reichhart, D., 2013. Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by *Saccharomyces cerevisiae*. Metab. Eng. 18, 25–35.

Gay, G., Debatud, J.C., 1987. Genetic study on indole-3 acetic acid production by ectomycorrhizal *Hebeloma* species: inter- and intraspecific variability in homo- and dikaryotic mycelia. Appl. Microbiol. Biotechnol. 26, 141–146.

Girhard, M., Machida, K., Itoh, M., Schmid, R.D., Arisawa, A., Urlacher, V.B., 2009. Regioselective biooxidation of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. Microb. Cell Fact. 8, 36.

Gounaris, Y., 2010. Biotechnology for the production of essential oils, flavours and volatile isolates. A review. Flavour Fragr. J. 25, 367–386.

Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. J. Hazard. Mater. 169, 1–15.

Hiscox, J., Hibbet, C., Rogers, H.J., Boddy, L., 2010. Monokaryons and dikaryons of *Trametes versicolor* have similar combative, enzyme and decay ability. Fungal Ecol. 3, 347–356.

Kaur, J., Sodhi, H.S., Kapoor, S., 2008. Breeding of *Pleurotus florida* (oyster mushroom) for phenotypic pigmentation and high yield potential. J. Sci. Food Agric. 88, 2676–2681.

Kothe, E., 2001. Mating-type genes for basidiomycete strain improvement in mushroom farming. Appl. Microbiol. Biotechnol. 56, 602–612.

Krings, U., Berger, R.G., 2010. Terpene bioconversion – how does its future look? Nat. Prod. Commun. 5, 1507–1522.

Krügener, S., Krings, U., Zorn, H., Berger, R.G., 2010. A dioxygenase of *Pleurotus sapidus* transforms (+)-valencene regio-specifically to (+)-nootkatone via a stereo-specific allylic hydroperoxidation. Bioresour. Technol. 101, 457–462.

Linke, D., Bouws, H., Peters, T., Nimtz, M., Berger, R.G., Zorn, H., 2005. Laccases of *Pleurotus sapidus*: characterization and cloning. J. Agric. Food Chem. 53, 9498–9505.

Linke, D., Nimtz, M., Berger, R.G., Zorn, H., 2009. Separation of extracellular esterases from pellet cultures of the basidiomycete *Pleurotus sapidus* by foam fractionation. J. Am. Oil Chem. Soc. 86, 437–444.

Lomascolo, A., Record, E., Herpoël-Gimbert, I., Delattre, M., Robert, J.L., Georis, J., Dauvrin, T., Sigoillot, J.C., Asther, M., 2003. Overproduction of laccase by monokaryotic strain of *Pycnoporus cinnabarinus* using ethanol as inducer. J. Appl. Microbiol. 94, 618–624.

Mattanovich, D., Branduardi, P., Dato, L., Gasser, B., Sauer, M., Porro, D., 2012. Recombinant protein production in yeasts. Methods Mol. Biol. 824, 329–358.

Olson, A., 2006. Genetic linkage between growth rate and the intersterility genes S and P in the basidiomycete *Heterobasidion annosum* s.lat.. Mycol. Res. 110, 979–984.

Omarini, A., Lechner, B.E., Alberto, E., 2009. *Polyporus tenuiculus*: a new naturally occurring mushroom that can be industrially cultivated on agricultural waste. J. Ind. Microbiol. Biotechnol. 36, 635–642.

Onken, J., Berger, R.G., 1999. Effects of R-(+)-limonene on submerged cultures of the terpene transforming basidiomycete *Pleurotus sapidus*. J. Biotechnol. 69, 163–168.

Plagemann, I., Zelena, K., Arendt, P., Ringel, P.D., Krings, U., Berger, R.G., 2012. LOX_{Psa1}, the first recombinant lipoxygenase from a basidiomycete fungus. J. Mol. Catal. B Enzym. 87, 99–104.

Rottava, I., Cortina, P.F., Grando, C.E., Colla, A.R.S., Martello, E., Cansian, R.L., Toniazzo, G., Treichel, H., Antunes, O.A.C., Oestreicher, E.G., de Oliveira, D., 2010. Isolation and screening of microorganisms for R-(+)-limonene and (−)-beta-pinene biotransformation. Appl. Biochem. Biotech. 162, 719–732.

Schuurs, T.A., Dalstra, H.J., Scheer, J.M., Wessels, J.G., 1998. Positioning of nuclei in the secondary mycelium of *Schizophyllum commune* in relation to differential gene expression. Fungal Genet. Biol. 23, 150–161.

Tello, M., Seelenfreund, D., Lobos, S., Gaskell, J., Cullen, D., Vicuna, R., 2001. Isolation and characterization of homokaryotic strains from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. FEMS Microbiol. Lett. 199, 91–96.

Uenojo, M., Pastore, G.M., 2010. Beta-Carotene biotransformation to obtain aroma compounds. Cienc. Tecnol. Aliment. 30, 822–827.

van der Nest, M.A., Slippers, B., Steenkamp, E.T., De Vos, L., Van Zyl, K., Stenlid, J., Wingfield, M.J., Wingfield, B.D., 2009. Genetic linkage map for *Amylostereum areolatum* reveals an association between vegetative growth and sexual and self-recognition. Fungal Genet. Biol. 46, 632–641.

Zelena, K., Krings, U., Berger, R.G., 2012. Functional expression of a valencene dioxygenase from *Pleurotus sapidus* in *E. coli*. Bioresour. Technol. 108, 231–239.