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Production of semi-biosynthetic nepetalactone in yeast

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Abstract

Microbial-based production of natural products provides a promising alternative to synthetic production and isolation from the native producer. The recently discovered NEPS1 cyclase/oxidase completes the biosynthetic pathway to nepetalactone, a biologically relevant iridoid known as both an insect repellent and cat attractant. In this work, we employ yeast based whole-cell biocatalysis to produce semi-biosynthetic nepetalactone from a low-cost precursor via a four-step enzymatic process. The dependence of product yield on bioprocess parameters ranging from induction of gene expression to substrate loading was investigated. Subsequent factorial design and response surface methodology optimization approach enabled a 5.8-fold increase in nepetalactone titer to 153 mg/L. Our study provides insights into strategies for operating plasmid-based bioconversion of a fed substrate and sets the stage for scalable, microbial synthesis of nepetalactone.

Introduction

Whole-cell biocatalysis offers unique advantages over traditional methods of chemical production and product extraction [14]. This is because microbial transformations using enzymes can perform difficult chemical reactions at mild operating conditions, with efficiencies and selectivities that are often superior to chemical syntheses. Additionally, if a target compound is acquired from a native producer that produces related cogeners, the use of a heterologous organism for production can often provide a means of affording a single constituent typically isolated as a complex mixture. Efforts towards microbial biosynthesis of plant natural products have gained increasing attention given recent advances in synthetic biology and metabolic engineering technologies [4]. In particular, the elucidation of biosynthetic routes towards medicinally useful plant natural products has been greatly accelerated by next generation transcriptomics and proteomics, leading to the successful heterologous production of several prominent compounds [7, 15].

The iridoids comprise a diverse family of biologically active natural products with reported activities ranging from anticancer to antibiotic, prompting clinical interest in iridoids and making them a value-added chemical target [17]. Recently, we established benchmark production of iridoids in *Saccharomyces cerevisiae* (Baker's yeast) using whole-cell

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biocatalysis starting from the low-cost substrate 8-hydroxygeraniol [2]. Combining shunt-pathway elucidation and metabolic engineering, fermentation-based production of the iridoid *cis-trans*-nepetalactol (referred to hereafter as “nepetalactol”) was demonstrated at a titer of 45 mg/L. Nepetalactol serves as the monoterpene scaffold for all monoterpene indole alkaloids, including the vinca alkaloids which have essential clinical utility as chemotherapeutics.

The recent discovery of NEPS1 by the O’Connor group provided the key enzyme required for biosynthesis of *cis-trans*-nepetalactone (referred to hereafter “nepetalactone”) in the catmint *Nepeta mussinii* [13]. NEPS1 was shown to catalyzes the oxidation of nepetalactol to nepetalactone. This iridoid compound is the primary active ingredient in products containing catnip essential oil, which exhibit an array of biological applications ranging from cat attractants to insect repellents. Additionally, consumer trends towards environmentally friendly products have been reported in both the natural insect repellent market ($550 million annual global sales [10]) and cat toy market ($260 million annual global sales [6]). Current nepetalactone production methods rely on catmint farming and steam distillation, which are both considered energy intensive processes.

The discovery of NEPS1 therefore provides an opportunity to investigate the versatility of our engineered iridoid-producing yeast strain as a “plug-and-play” host for production of a commercially relevant iridoid natural product. Herein, we describe our approach which enabled production of nepetalactone. Addition of NEPS1 to our existing strain enabled the immediate production of nepetalactone at 26 mg/L. Next, our strategy involved identification of the variables which likely affected the product tier, and a systematic one-factor-at-a-time optimization of each of these parameters. Multivariate optimization of critical bioconversion parameters was then carried out using a factorial design and response surface methodology to further improve operating conditions. This strategy led to a 5.8-fold increase in product titer to 153 mg/L and provided insights into bioprocess optimization using yeast as a chassis for value-added bioconversion.

Results and Discussion

Implementation of NEPS1.

Previously, we engineered a yeast strain capable of producing the nepetalactone precursor, nepetalactol, from the supplied substrate 8-hydroxygeraniol [2]. A facile chemical synthesis of 8-hydroxygeraniol from geranyl acetate was recently reported, resulting in improved economic feasibility of this bioprocess [9]. Production of nepetalactol required expression of the NAD^+-dependent geraniol oxidoreductase (GOR) and the NAD(P)H-dependent iridoid synthase (ISY) (Fig. 1A). The bioconversion platform consists of a strain-plasmid pair, consisting of a platform strain which has been engineered for improved metabolic flux and a plasmid which encodes a biosynthetic pathway. This method allows strain engineering to be performed independently of pathway design. Previously, expression of GOR and/or ISY in the wild type yeast strain allowed for the elucidation of two metabolic routes to a dead-end shunt pathway. Strain engineering was performed in order improve the selectivity towards nepetalactol more than 5-fold, which involved deletion of yeast old yellow enzymes and alcohol dehydrogenases [2].

Here, we sought to expand our platform's capability by including the newly discovered downstream enzyme, NEPS1. This enzyme was recently shown to assist in both the stereoselective cyclization and subsequent oxidation to provide nepetalactone (Fig. 1A). Oxidation of the labile hemiacetal in nepetalactol to a lactone by NEPS1 provides a more stable final product not prone to ring-opening or epimerization, facilitating both metabolite analysis and purification of the biocatalyst product. An expression cassette for codon optimized NEPS1 from *Nepeta mussinii* was synthesized for compatibility with the existing promoters and terminators. The recently characterized MLS1 promoter [8] and SPG5 terminator [4] were selected for coordinated gene expression. These genetic elements have approximately equal induction and/or expression profiles to the promoters and terminators driving GOR and ISY expression. The expression cassette was cloned downstream of GOR and ISY using yeast homologous recombination methods, generating the plasmid pJB133 (Fig. 1B), and transformed into the yeast strain YJB051. A standard single fed-batch bioconversion was performed, and production of nepetalactone at 26 mg/L was observed via GC-MS, which corresponds to a yield of 0.08 (actual/theoretical). As observed previously with the yeast platform, approximately 75% of the fed substrate was converted to the 8-hydroxytetrahydrogeraniol shunt product.

The iridoid profile from the whole-cell bioconversion was compared to extracts from two leading catnip products, Yeowww! Catnip and KONG Naturals Catnip Spray (Fig. 1C), as described previously [2]. GC-MS profiles for the two commercial nepetalactone sources indicated a mixture of metabolites evidenced by multiple overlapping chromatogram peaks with $m/z = 166$ corresponding to the characteristic nepetalactone parent ion. A number of plants produce mixtures of iridoids, varying in stereochemistry at the bridgehead carbons [1]. Meanwhile, our yeast strain was able to specifically produce the *cis-trans*-nepetalactone stereoisomer, with a retention time and fragmentation pattern matching the authentic standard. This result highlights the ability to produce a single constituent within a complex mixture as a major advantage offered by microbial biosynthesis.

One-factor-at-a-time optimization.

In recent years, production and discovery of small molecules in yeast have utilized late-stage inducible promoters including ADH2p, PCK1p, and MLS1p. Late-stage inducible promoters are repressed during exponential growth in standard dextrose-containing yeast media, alleviating growth defects that are often observed in strains engineered for constitutive expression [5]. Upon depletion of glucose, the promoters are rapidly and strongly induced, obviating the need for addition of a small-molecule inducer. Thus, utilization of late-stage inducible promoters can simplify bioprocesses, but introduces growth dependent, and therefore time-dependent gene induction. Accordingly, a careful tuning is required in order to balance gene induction with cellular respiration to ensure that the enzymes are sufficiently active. In addition to timing of substrate addition, several other bioprocess parameters have been shown to impact bioconversion titer. For example, the concentration of yeast cells in the system is known to affect the efficiency of conversion, and a common laboratory practice for small scale culturing is to concentrate the yeast cells prior to substrate addition [11]. Furthermore, a repeated fed-batch feeding schedule was previously employed to improve iridoid yield [2], however the individual effects of timing and amount of substrate added

were not explicitly investigated. Additionally, since our bioconversion platform uses a plasmid-based gene expression system, an initial seed culture in selective media is grown prior to inoculation in nonselective rich media. While inoculants grown in selective media are commonly employed in bioprocesses, the effects of the duration of this preculture period in our system has not been explored.

Towards establishing the impact of each of the variables on nepetalactone production, we performed a one-factor-at-a-time investigation of the aforementioned factors on product titer (Fig. 2). The selected variables include duration of preculture in selective media (t_1), duration of culture in nonselective media (t_2), duration of bioconversion (t_3), total number of fold concentration of cells (n_1), number of substrate additions (n_2), and amount of substrate added as determined by the mass fraction of the substrate in an ethanol stock solution (x). All bioconversion experiments were performed at small scale using culture tubes as described previously [2]. Each factor was individually varied, and the resulting nepetalactone production was measured via GC-MS. Results of our initial analysis are shown in Fig. 2.

As expected, variation of all six of the bioconversion variables led to different nepetalactone titers. Of the parameters investigated, variation of preculture duration and fold concentration provided only marginal increases in peak area (1% and 3% increase, respectively). Accordingly, in successive experiments, we elected to select conditions for t_1 and n_1 in order to reduce the duration and complexity of the overall bioprocess. By operating at a $t_1 = 12$ hours, we could reduce the experimental duration of this variable by half. Since the total culturing duration is equal to $t_1 + t_2 + t_3$, a 50% reduction in t_1 provides substantial improvement in the overall productivity ($mg*L^{-1}*h^{-1}$). Similarly, operating at $n_1 = 1$ enables the cells from the YPD culture to be used directly, eliminating an entire unit operation (centrifugation for concentration) from the overall bioprocess. The remaining four parameters, t_2, t_3, x, and n_2, provided maximum increases in nepetalactone peak area of 17%, 29%, 251%, and 44%, respectively.

While the maxima observed in Fig. 2 are approaching local optima, our one-factor-at-a-time optimization provides valuable information about the properties of our biocatalyst. For example, as shown in Fig. 2B, the data indicates that a 12 hour preculture in selective media provides sufficient plasmid stability in our single plasmid system, and that increasing this duration would lower the overall productivity. Similarly, a 12-hour bioconversion phase was shown to be sufficient for maximal substrate consumption, and an extended bioconversion duration led to a decrease in product titer as in indicated in Fig. 2D, potentially through product evaporation or product degradation. On the other hand, our data show that several variables must be substantially adjusted to provide improved product titers. For example, increased substrate loading provided the most dramatic increase in product titer, however increasing the substrate mass fraction (x) too much resulted in a decrease in titer. Indeed, a decrease in the relative nepetalactone peak area per milligram substrate fed accompanies the titer improvements when increasing substrate loading (Supplementary Fig. 2). The decrease in yield could be caused by substrate toxicity, which could be alleviated by adjusting the 8-hydroxygeraniol feeding schedule. Additionally, a 3-fold increase in the YPD culture duration from 24 hours to 72 hours provided the highest relative nepetalactone peak area

when varying t_3. This result was unexpected, since previous investigations indicated maximal induction of ADH2-like promoters after 24 hours [8, 12]. This result highlights the context-dependent behavior of genetic parts as well as the shift in metabolic properties of our biocatalyst over time, and emphasizes the need for a multivariate optimization approach.

Factorial design and response surface methodology optimization.

In contrast to one-factor-at-a-time optimization, multivariate optimization methods enable rapid global optimization of complex systems with many independent variables. Response surface methodology (RSM) has been commonly employed for optimization of biological processes [16]. One major advantage of the RSM approach is that a relatively small number of experiments may be carried out in order to assess how two variables may positively interact to produce a desired outcome. Generation of a response surface simultaneously allows for the approximation of a global optimum.

As determined in earlier experiments, the parameters, t_2, t_3, x, and n_2 were shown to have the greatest effect on the response variable (nepetalactone peak area). Design-Expert 11 was employed for Design of Experiments (DoE) and subsequent generation of the response surface. Coded levels for the four independent variables are shown in Supplementary Table 1. All bioconversion experiments were performed at small scale using culture tubes as described previously [2]. A total of 73 trials defined by Design Expert and shown in Supplementary Table 2 were performed, and the nepetalactone peak area was assessed using GC-MS as described previously [2]. Several conditions resulted in no observable nepetalactone peak, resulting in a response value of zero. These conditions corresponded to the lowest coded values for either “x” or “t_3”. Results of quadratic response surface model fitting using ANOVA and other statistical parameters are given in Supplementary Table 1. The model was determined to be significant ($p<0.0001$) with values for R^2 of 0.794 and a signal-to-noise of 13.6, despite a substantial lack-of-fit. We concluded that this model was suitable for navigation of the design space.

Numerical optimization indicated that the maximum coded values of $t_{3,optimal}$ = 12 hours and $n_{2,optimal}$ = 12 times provided the greatest product titer. While this result could be predicted by our previous optimization of t_3, the requirement for increased number of substrate additions is only apparent when operating with increased substrate concentration. On the other hand, the model predicted maximal nepetalactone titers of $t_{2,optimal}$ = 66 hours and $x_{optimal}$ = 0.45, which fell within the coded values. The predicted value for $t_{2,optimal}$ is consistent with the value observed previously, while $x_{optimal}$ is substantially higher than expected. We reason that the increased mass fraction “x” is permitted via synergistic improvements provided by varying other factors in the feeding schedule, which were not captured during one-factor-at-a-time optimization. The two-variable response surface for t_2 and x, where t_3 = 12 hours and n = 12 times, is shown in Fig. 3A.

Finally, in order to validate the model’s ability to predict improved nepetalactone titer, the bioconversion assay was performed using the optimized t_1, t_2, t_3, n_1, n_2, and x. A titer of 153 mg/L was obtained, corresponding to a 5.8-fold increase in nepetalactone titer (Fig. 3B). While this value represents a benchmark titer for microbial iridoid production, it should be noted that a 50% decrease in the overall yield was observed. This may be attributed to low

conversion and selectivity, which could be optimized in subsequent RSM studies. The nepetalactone cost per gram is approximately 25 times greater than that of synthesized 8-hydroxygeraniol and 500 times greater than that of the process feedstock geranyl acetate. Further, approximately 75% of the substrate remained unconsumed, which could also be extracted and recycled to further improve process efficiency. While the optimized process parameters at this scale cannot be directly applied to industrial-scale fermentation, response surface methodology is a general approach which could be utilized to for bench-scale fermentation optimization. Ultimately, factors such as purification requirements, substrate supply, and scalability must be considered to further refine operating conditions.

Conclusions

In this study, the recently discovered NEPS1 was employed for semi-biosynthetic nepetalactone production in *Saccharomyces cerevisiae* using whole-cell biocatalysis. Both one-factor-at-a-time and multivariate optimizations of the bioconversion process parameters were carried out; execution of predictions from the RSM method led to a 5.8-fold increase in product titer. Our process provides an applicable method for species and climate independent access to nepetalactone at 153 mg/L.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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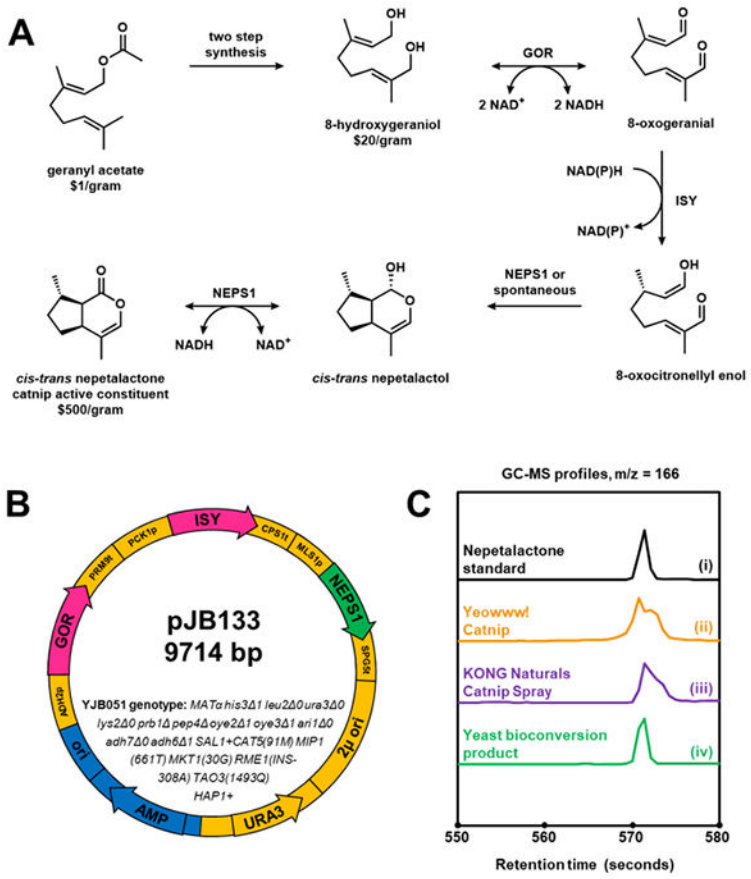

Fig. 1.

Strategy for production of nepetalactone in yeast. A) Synthesis and of 8-hydroxygeraniol from geranyl acetate and conversion to nepetalactone involving geraniol oxidoreductase (GOR), iridoid synthase (ISY), and nepetalactol-related short-chain dehydrogenase enzymes (NEPS1). B) Nepetalactone GC-MS profiles (m/z = 166) comparing (i) an authentic standard, (ii, iii) leading catnip containing products, and (iii) the bioconversion product of our yeast strain transformed with pJB133.

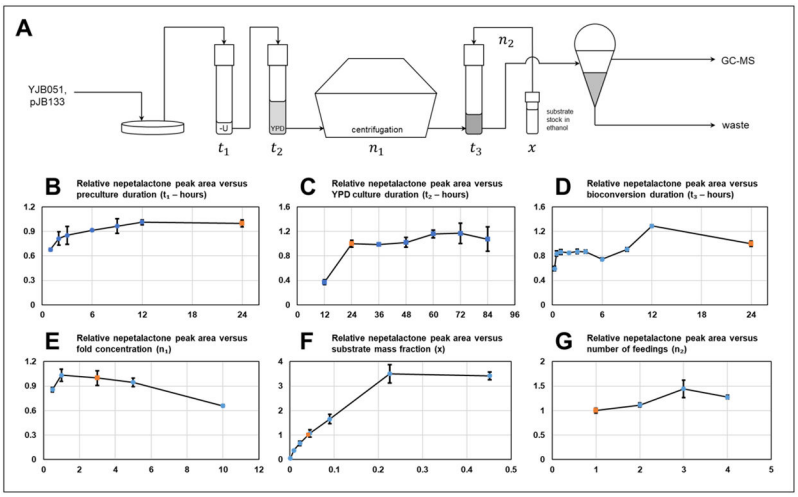

Fig. 2.

Process flow diagram of 8-hydroxygeraniol bioconversion (A) as well as one-factor-at-a-time investigation of bioconversion parameters (B-G), including preculture duration (t_1), YPD culture duration (t_2), bioconversion phase duration (t_3), total number of fold concentration of cells (n_1), substrate additions (n_2), and amount of substrate added as determined by the mass fraction of the substrate stock solution (x). Orange squares correspond to the unoptimized bioprocess.

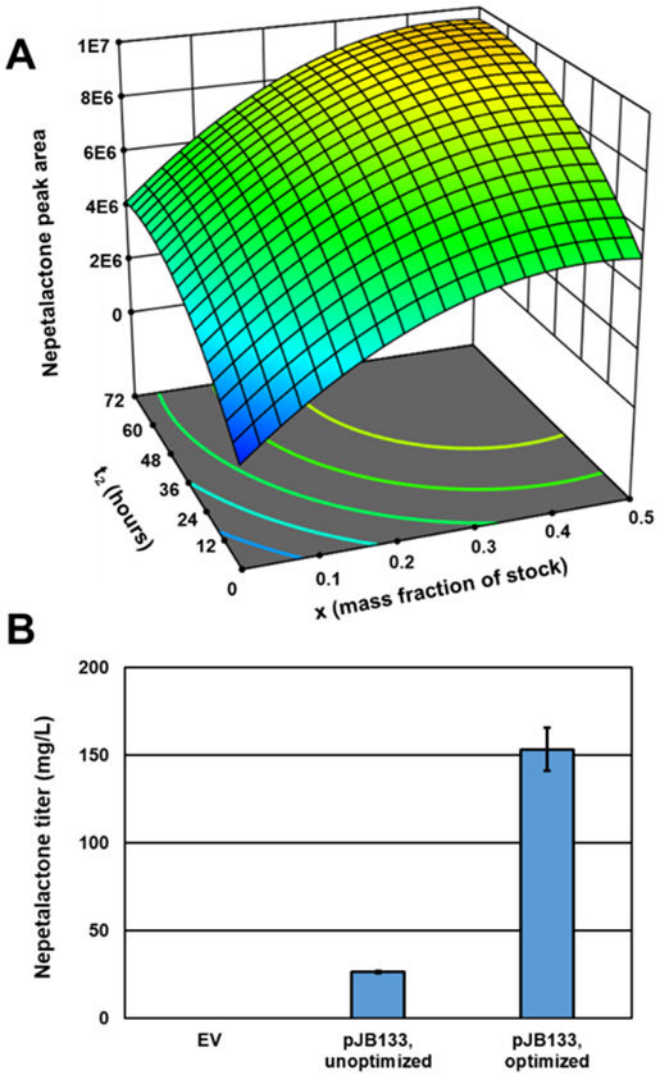

Fig. 3.

Optimization of bioprocess parameters for improved nepetalactone titer. (A) The two-variable response surface for t_2 and x, where t_3 = 12 hours and n = 12 times. (B) Plasmid map of pJB133 and genotype of YJB051. The source of all genetic parts used for plasmid construction is indicated by color and detailed in Supplementary Fig. 1. (C) Product titer for empty vector (EV) compared to unoptimized and optimized pJB133 bioconversion.