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Short communication

Antimicrobial and antioxidant activities of phenolic metabolites from flavonoid-producing yeast: Potential as natural food preservatives

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ABSTRACT

We analysed the antimicrobial and antioxidant activities of phenolic metabolites secreted from a naringenin-producing *Saccharomyces cerevisiae* strain (a GRAS organism), against the pure flavonoid naringenin and its prenylated derivatives, to assess their potential as natural food preservatives. Agar disc diffusion assay was used to analyse the antimicrobial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213, while DMPD· chemiluminescence assay was used to analyse antioxidant activity, based on DMPD·$^+$-scavenging activity. Our results showed that the engineered yeast metabolites exhibited both strong antimicrobial and DMPD·$^+$-scavenging activity, particularly the metabolite phenylacetaldehyde. Pure naringenin had poor antimicrobial and DMPD·$^+$-scavenging effects. Prenylated varieties, 6-prenylnaringenin and 8-prenylnaringenin, inhibited only *S. aureus,* while only 8-prenylnaringenin exhibited moderate DMPD·$^+$-scavenging activity. Our results suggested that phenolic metabolites secreted from naringenin-producing yeast would be a sustainable source of natural food preservatives.

1. Introduction

Food preservatives are substances added to food to slow or prevent food spoilage caused by microbes or oxidation. Modern food preservatives are usually synthetic chemicals, such as sorbates, benzoates, nitrates and nitrites (Russell, 1991; Silva & Lidon, 2016). However, more research in recent times have begun to shed light on the non-negligible health risks of consuming these synthetic food additives even below recommended limits as defined by regulatory agencies, such as the FDA. These health risks include allergic reactions, gastrointestinal disorders and cancer (Etemadi et al., 2017; Varraso & Camargo, 2014). There is therefore much ongoing interest in more "natural" sources of food preservatives, which can be plant extracts, essential oils or purified secondary metabolites (Carocho, Barreiro, Morales, & Ferreira, 2014; Gassara, Kouassi, Brar, & Belkacemi, 2016).

Flavonoids, belonging to the phenolic class of plant secondary metabolites, are distinguished from the other phenolics by their 15-carbon, C_6-C_3-C_6 (aryl-propyl-aryl) structural backbone. They are primarily responsible for the vivid colours of flowering plants. Aside from colour pigments, flavonoids are typically produced in plants in response to environmental changes, usually as a defence mechanism, e.g. UV exposure, pathogenic invasion (Winkel-Shirley, 2001). The flavonoid biosynthetic pathway occurs mainly in the plant cell cytosol, with some downstream ancillary enzymes compartmentalized in plastids. There is a tremendous and still-growing body of literature on the significant health benefits of flavonoids, spanning decades. These include antioxidant, anti-inflammatory, anticancer, antimicrobial and antidiabetic properties (Araya-Cloutier, Vincken, van Ederen, den Besten, & Gruppen, 2018; Falcone Ferreyra, Rius, & Casati, 2012; Sharma, Gupta, Sarethy, Dang, & Gabrani, 2012; Sun et al., 2017; Tunon, Garcia-Mediavilla, Sanchez-Campos, & Gonzalez-Gallego, 2009; Venturelli et al., 2016).

The widespread occurrence of flavonoids in plants naturally extends to the human diet as well. Daily total consumption of flavonoids may exceed 1 g (Zamora-Ros et al., 2016), with major contributing sources being fruits and vegetables, and beverages, such as tea, cocoa, wine and beer. Commercial applications of flavonoids include food additives, nutraceuticals, pharmaceuticals, cosmetics and others. Global market and demand for flavonoids was valued at over 840 million USD in 2015 and is expected to surpass 1 trillion USD beyond 2020 (https://www.zionmarketresearch.com/news/global-flavonoids-market). Currently, options for large-scale commercial production of flavonoids for nutraceutical, pharmaceutical and functional food applications are plant extraction and chemical synthesis. Plant extraction remains the default method, but carries significant downsides of high cost and significant carbon footprint, due to excessive energy and solvent requirements

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(Guo-qing et al., 2005). Chemical syntheses have similar disadvantages, with the additional hurdle of poor product stereoselectivity (De Luca, Salim, Atsumi, & Yu, 2012). Microbial biofermentation of metabolically engineered microbes has been touted as a superior alternative to both methods and has garnered increasing attention in recent times, due to several reasons. Firstly, the lower complexity of culture suspensions relative to solid plant matrices enables easier and less costly separation techniques with lower carbon footprint (Ameer, Shahbaz, & Kwon, 2017; Azmir et al., 2013). Secondly, plant extraction production levels are gated by plant supply, which may be highly seasonal depending on the specific crop, whereas microbial production has no such seasonal reliance owing to very short life cycles in the metric of days, and also only requiring the initial input of simple feedstock, such as complex nutrient media with simple carbon sources, e.g. glucose and oxygen and/or carbon dioxide, and will therefore be more reliable in meeting production demand. Thirdly, the established knowledge of common microbial workhorses, such as *Escherichia coli* and *Saccharomyces cerevisiae*, plus the plethora of bioengineering tools at our disposal currently allows for high customizability, versatility and targetability, from *in silico* design to production of target compounds. Plants in contrast, accumulate these different secondary metabolites to highly variable extents, usually yielding a range of flavonoids and their derivatives. Moreover, high-resolution separation of a complex mixture of such closely related compounds poses further challenges and cost. Therefore, whole plants are less "production-efficient" when compared to microbial cell platforms. Lastly, engineered microbial cell factories allow waste valorization (Ong, Kaur, Pensupa, Uisan, & Lin, 2017; Widsten, Cruz, Fletcher, Pajak, & McGhie, 2014), which further cheapens production while promoting a perfectly circular economy.

Plant extracts containing flavonoids, such as naringenin, have reportedly demonstrated antimicrobial activities (Mandalari et al., 2007; Nowak, Czyzowska, Efenberger, & Krala, 2016; Rauha et al., 2000). Allied with their inherently potent nutraceutical benefits, the use of extracts containing flavonoids as novel food preservatives would therefore be highly desirable and profitable.

The objective of our study was to investigate the antimicrobial and antioxidant effects of microbial phenolic compounds from naringenin-producing *S. cerevisiae* (N2) and compare that against the bioactivities of pure flavonoids naringenin (NAR) and its prenylated derivatives 6-prenylnaringenin (6PN) and 8-prenylnaringenin (8PN), for evaluation of their potential as natural substitutes for synthetic food preservatives.

2. Materials and methods

2.1. Reagents, media and strains

Naringenin-producer *Saccharomyces cerevisiae* strain Y26 (herein renamed N2) was engineered based on our previous work (Lyu, Ng, Lee, Mark, & Chen, 2017). *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *S. cerevisiae* BY4741 (wild-type) were kindly provided by the School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore. 6-prenylnaringenin and 8-prenylnaringenin (both 99%) were purchased from BOC Sciences, USA. The phenolics naringenin, phloretic acid, phenylacetaldehyde and homogentisic acid were procured from Sigma Aldrich, USA. N,N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD), sodium acetate trihydrate and ferric chloride hexahydrate were also procured from Sigma Aldrich, USA. Luria-Bertani (LB) broth formula, ethyl acetate (HPLC-grade) and ethyl alcohol (> 99%) were procured from Sigma Aldrich, USA. Mueller-Hinton Broth formula was purchased from Fisher Scientific, USA.

2.2. Shake-flask cultivation of yeast

Strains N2 and BY4741 wild-type were first separately cultured overnight in 5 ml of YPD medium at 30 °C with shaking (200 rpm). This overnight seed culture was then inoculated into 50 ml fresh YPSG (1% sucrose-1% glycerol) media to an initial OD_{600} of 0.05 and incubated under the same conditions for another 72 h.

2.3. Extraction, HPLC and LC–MS analysis of samples

After completion of the shake flask cultures, the supernatants were harvested for phenolic extraction and quantification. Both engineered (N2) and BY4741 negative control (WT) *Saccharomyces cerevisiae* cultures were processed as follows: firstly, centrifugation was performed at 10,000 rpm to separate and remove the cell pellet. An equal volume of ethyl acetate was then added to the supernatant (1:1 aqueous:organic solvent), vortexed vigorously for 30 s, and rotated at room temperature overnight. After another centrifugation at 10,000 rpm for 10 min, the upper organic layer was collected and 500 µl set aside and microfiltered (0.45 µm) for HPLC analysis and quantification. The rest of the ethyl acetate extract was evaporated to dryness, leaving behind a concentrated extract residue. Quantification of naringenin in extracts was performed through HPLC (Agilent 1100) equipped with a variable wavelength detector and C18 column (4.6 mm × 150 mm, RESTEK). Samples were analysed using a gradient method. The program started with 25% of solvent A (methanol) and 75% of solvent B (water). The concentration of A subsequently increased to 75% within 10 min, continued up to 100% at 20 min, and then was held for 10 min. The solvent was returned to 25% A over 2 min and held for 13 min. The flow rate was 0.5 ml/min, and the signal was detected at 280 nm. A pre-established calibration curve using naringenin standards was used for quantification. After HPLC analysis and quantification, the extract residue was re-dissolved in an appropriate amount of 100% ethanol to obtain the equivalent of 40 mg/ml concentration of naringenin in ethanol, which served as a stock solution for use in further assays. The BY4741 wild-type culture extract (WT) – containing no flavonoids or phenolics – was prepared identically to the N2 extract as a negative control.

Identification of other phenolic metabolites in extracts was performed through LCMS. Samples were analysed with an Agilent 6550 iFunnel Q-TOF LC–MS using an injection volume of 2 µl. Samples were separated on an Agilent Zorbax SB-C18 column (2.1 × 100 mm, 1.8 µm) with the guard column at 30 °C. The mobile phases consisted of (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. The separation was performed using the following gradient: 0 min at 5% B, 8–10 min at 95% B, 10.1–13 min at 5% B at a flowrate of 0.2 ml/min. Analysis was done using an Agilent Jet Stream dual electrospray in ionization negative mode, with drying gas temperature of 200 °C, drying gas flow of 14 l/min, nebulizer pressure of 35 psig, sheath gas temperature of 350 °C and sheath gas flow of 11 l/min. MS Scan was in the range of *m/z* 100–800. A reference solution containing mass 112.985587 was constantly infused as an accurate mass reference. Chromatogram and MS results were subject to METLIN database search (http://metlin.scripps.edu). Metabolites were singled out based on significant peak height, area and score of > 75. Compound hits that did not meet designated criteria were excluded. Hits that were not unique to N2 (present in both WT control and N2) were also excluded.

2.4. Antimicrobial assay (agar disc diffusion)

The antimicrobial activity of the extract was surveyed by employing the disc diffusion assay, to determine the inhibition zones of all samples against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 separately. Each strain was first streaked on fresh LB agar plates and incubated at 37 °C overnight. From the overnight plate, individual colonies were then re-suspended in 1 ml Mueller-Hinton (MH) broth such that the OD_{600} value approximated 0.5 McFarland standard, corresponding to approximately 1–3 × 10^8 CFU/ml. A sterile cotton swab was used to streak-inoculate each fresh MH agar plate. N2 yeast extract stock solution (40 mg/ml naringenin equivalent in ethanol), wild-type