Bioconversion of isoeugenol to vanillin and vanillic acid using the resting cells of *Trichosporon asahii*

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Abstract 40 isoeugenol-tolerant yeasts were isolated from the rhizosphere soil samples which in turn were collected from aromatic plants in different regions of Iran, and further tested for their ability to grow on a minimal medium containing isoeugenol as the sole carbon and energy source. Nine isolates which were able to grow on isoeugenol were examined for their ability to convert isoeugenol into vanillin under growing cell experiments. Of the tested yeasts, the highest conversion efficiency was observed in isolate MP24. The isolate was identified as *Trichosporon asahii* based on morphological, biochemical and molecular (ITS region) characters and tested to effectively convert isoeugenol into vanillin under resting cell system. A comparative analysis of thin layer chromatography (TLC), UV–Vis spectrometry, and high-performance liquid chromatography (HPLC) verified that vanillin and vanillic acid are accumulated as two major metabolites using *T. asahii* strain MP24 resting cells. In the presence of 7.5 g/l of wet weight cells of the strain MP24 pre-grown on isoeugenol and harvested at the end of the exponential growth phase, the optimal concentration of vanillin reached 2.4 g/l with a molar conversion of 52.5% in the potassium phosphate buffer (100 mM, pH 5.8) supplemented with 5 g/l of isoeugenol and 2% (v/v) *N,N*-dimethylformamide (DMF). The total concentration of vanillin and vanillic acid obtained from the bioconversion process was 4.2 g/l (total

molar yield of 88.3%). Until now, no data has been published on the conversion of isoeugenol into vanillin by the strains of the genus *Trichosporon*.

Keywords Bioconversion · Isoeugenol · Resting cell · *Trichosporon asahii* · Vanillin

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a chemical compound with many applications in nutritional, cosmetic, pharmaceutical, medical, agricultural and various other industries (Vyrides et al. 2015). It is widely used as perfume and flavour enhancer in many food and beverage items including, cakes, chewing gums, chocolates, ice-creams, muffins, and carbonated or non-carbonated beverages (Mathew and Abraham 2006). Vanillin is also used as a food preservative due to its antioxidant activities and antimicrobial properties (Fitzgerald et al. 2003). Furthermore, it is used as an intermediate in the chemical synthesis of herbicides and defoaming agents (Hocking 1997). Vanillin is also used in the pharmaceutical preparations, as a starting material for the production of drugs, such as Aldomet, dopamine and L-dopa (Walton et al. 2003). Natural vanillin derived from green vanilla beans is very expensive and in limited supply. Moreover, most of the vanillin sold commercially is produced by chemical synthesis, involving catalysts that are not environment-friendly. Hence, biotechnological production of vanillin is a cleaner and more economical alternative for the production of natural vanillin (Priefert et al. 2001). Many compounds can be used in natural vanillin production, such as eugenol, isoeugenol, ferulic acid, glucose, and tyrosine (Ni et al. 2015; Zhao et al. 2015; Fleige et al. 2016). In

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particular, bioconversion of propenylbenzenes using microbial catalysts is being investigated as a promising alternative for synthesizing high-value products, such as vanillin and vanillic acid (Xu et al. 2007). Isoeugenol (4-hydroxyl-3-methoxy-1-propenyl benzene) is a member of the propenylbenzenes, which can either be obtained using direct extraction of essential oils—especially from the clove tree *Syzygium aromaticum*—or obtained using the isomerization of eugenol (Zhang et al. 2006). Isoeugenol extracted from essential oils is a renewable precursor, and is widely used as an economic substrate to get natural vanillin. Thus, the conversion of isoeugenol into vanillin by certain microorganisms has long been considered as an attractive opportunity for vanillin production from the cheap precursor, which can be labelled as ‘natural’ according to USDA and European regulations (Serra et al. 2005). Due to the fact that vanillin has been detected as an intermediate in the catabolism of isoeugenol, the sustained extensive research has been developed during the last two decades for the preparation of natural vanillin through microbial conversion of isoeugenol. The first evidence on the bioconversion of isoeugenol into vanillin was reported in *Aspergillus niger* ATCC 9142. The low yield of vanillin (10%) was achieved due to further metabolization of vanillin to vanillic acid and vanillyl alcohol (Abraham et al. 1988). The conversion of isoeugenol into vanillin by numerous bacteria, including *Serratia marcescens* DSM30126 (Rabenhorst and Hopp 1991), *B. subtilis* B2 (Shimoni et al. 2000), *B. subtilis* HS8 (Zhang et al. 2006), *B. fusiformis* CGMCC1347 (Zhao et al. 2006), *B. pumilus* S1 (Hua et al. 2007), *Pseudomonas putida* IE27 (Yamada et al. 2007), *Psychrobacter* sp. strain CSW4 (Ashengroph et al. 2012), and *Lysinibacillus fusiformis* SW-B9 (Zhao et al. 2015), has been frequently reported. However, most of the mentioned processes result in rather low vanillin productivity due to the low solubility of isoeugenol in water (at most 1 g/l), its toxic effect, and product inhibition. In addition to resin, the free extract of cells, the manipulation of reaction parameters, solubility improvement, aqueous-organic system, resting cell strategy, and metabolic engineering were developed for achieving better yields (Table 1). The possibility of producing vanillin from isoeugenol was also investigated with the enzyme lipoxigenase (sigma L8383) and enzyme extracts from soya beans (Markus et al. 1992; Li et al. 2005). Nevertheless, the resultant vanillin yields were very low, with a bioconversion efficiency of only 11%. Yeasts are ubiquitous inhabitants of soil and aquatic environments, and they are promising candidates for a wide range of bioconversion processes due to their metabolic versatility, their relatively fast growth rate, their ease of downstream processing as well as due to their Generally Regarded as Safe (GRAS) (Kurtzman et al. 2011). This study aims to isolate and

identify isoeugenol-degrading yeasts with a potential of converting isoeugenol to vanillin. Here, we describe the results of the first evidence of the microbial production of vanillin and vanillic acid from isoeugenol by the newly isolated yeast *T. asahii* strain, MP24, under resting cell experiments (Fig. 1).

Materials and methods

Chemicals and media

The standards of isoeugenol (98%, *cis-trans* mixture), vanillin (99% purity), and vanillic acid (99% purity) were purchased from Sigma-Aldrich, UK, and used for bioconversion experiments. Chloramphenicol, rose bengal, peptone, and yeast extracts were procured from QuLab, Canada. HPLC-grade acetonitrile and DMF were acquired from Merck, Germany. Cycloheximide was obtained from Sigma-Aldrich, UK. Yeast Carbon Base (YCB) and Yeast Nitrogen Base (YNB) media were purchased from Difco, USA. All other chemicals used in the present study were of the analytical grade.

Screening of isoeugenol bioconversion culture

Rhizospheric soil samples for the isolation of isoeugenol-converting yeast strains were collected from farms or gardens for the cultivation of aromatic and medicinal plants in different regions of Iran. One gram of the collected samples was diluted in 100 ml of buffered peptone water (0.1% w/v peptone), and 0.2 ml of diluted soil (from 10^{-1} to 10^{-6}) was poured onto YPD plates (20 g/l glucose, 20 g/l peptone, 10 g/l yeast extracts, 20 g/l agar, pH 5.8) supplemented with 0.1 g/l of chloramphenicol to inhibit bacterial growth and 0.025 g/l rose bengal to restrict the growth of rapidly growing moulds. Isoeugenol was dissolved in 2% (v/v) DMF and added to the cultures to get a final concentration of 5 g/l. The plates were incubated under aerobic conditions at 28 °C for 48 h in the dark. Well-isolated colonies growing on the above media were selected and transferred onto the same fresh isolation media to obtain pure cultures of isoeugenol-tolerant yeasts. For isolating isoeugenol-degrading yeast cultures, an enrichment technique was used. A loop full of actively growing cultures (log phase) of the isoeugenol-tolerant yeasts was prepared and inoculated into 100 ml basal medium (pH 5.8) in 250-ml Erlenmeyer flask. The basal medium consisted of: NH_4Cl , 2.5 g/l; $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2.1 g/l; NaH_2PO_4 , 12.7 g/l; NaCl , 0.25 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l; and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1. Isoeugenol at final concentration of 5 g/l was added as a sole carbon and energy source. Cultures were incubated at 28 °C in incubator

Table 1 Comparison of studies reported the bioconversion of isoeugenol to vanillin by bacterial strains

Microorganism	Final molar yield (%)	Initial substrate	Reaction time (h)	Conversion system	References
<i>Serratia marcescens</i> DSM 30126	20.5	20 g/l	212	Growing culture	Rabenhorst and Hopp (1991)
<i>Rhodococcus rhodochrous</i> MTCC 289	58	1 g/l	72	Whole cell	Chatterjee et al. (1999)
<i>Bacillus subtilis</i> B2	14.0	1% (v/v)	48	Cell free extract	Shimoni et al. (2000)
<i>Bacillus subtilis</i> HS8	14.7	10 g/l	96	Whole cell	Zhang et al. (2006)
<i>Bacillus fusiformis</i> CGMCC 1347	17.4	50 g/l	72	Addition of 12.5 g HD-8 resin	Zhao et al. (2006)
<i>Bacillus pumilus</i> S1	40.5	10 g/l	150	Growing culture	Hua et al. (2007)
<i>Pseudomonas chlororaphis</i> CDAE5	12.6	10 g/l	24	growing culture	Kasana et al. (2007)
<i>Pseudomonas putida</i> IE27	71.0	150 mM (10% DMSO)	24	Resting cell	Yamada et al. (2007)
Recombinant <i>E. coli</i> BL21 (DE3)	81	230 mM (10% DMSO)	6	–	Yamada et al. (2008)
<i>Psychrobacter</i> sp. CSW4	13.8	10 g/l	48	Resting cell	Ashengroph et al. (2012)
<i>Lysinibacillus fusiformis</i> SW-B9	5.83	60% (v/v)	72	Aqueous-organic system	Zhao et al. (2015)

Fig. 1 Biological conversion of isoeugenol to vanillin and vanillic acid using the resting cells of *Trichosporon asahii*

shaker at 200 rpm for 48 h. After three transfers, cultures were diluted and placed on YPD agar plates, followed by 24–48 h incubation at 28 °C, and single colonies were picked and used for isoeugenol conversion screening. To screen isoeugenol-degrading yeasts with potential conversion of isoeugenol into vanillin under growing cultures, the cells were grown in 100-ml of YPD broth media in 250-ml of Erlenmeyer flasks and incubated at 28 °C in a rotary shaker (200 rpm) for 24 h. After this, isoeugenol, at final concentration of 5 g/l was added. 24 hours after adding isoeugenol, the samples were collected and centrifuged (10,000 × *g* for 10 min). The reaction products were acidified with aqueous hydrochloric acid and extracted with chloroform-collected organic fractions derived over anhydrous sodium sulphate by the rotary evaporator (Laborota 4010, Heidolph, Germany). The extracts were then subjected to the TLC, UV–Vis spectrophotometer, and HPLC analysis to check for isoeugenol bioconversion.

Simultaneously, control experiments were carried out without microorganisms under the same culture conditions.

Characterization of strain MP24

The strain MP24, which shows the maximum bioconversion of isoeugenol into vanillin, was characterized. A microscopic investigation was carried out under light microscopy (Olympus BX40, Japan). Physiological and biochemical tests were performed using the methods described by Kurtzman et al. (2011). The assimilation patterns of carbon and nitrogen compounds with cultures on YNB (Difco) and YCB (Difco) were determined. The ability to hydrolyse urea, growth at various temperatures with cultures on the YPD agar, and additional growth tests on the YNB media with 0.5% glucose containing 0.01 or 0.1% cycloheximide were also studied. The tests were done in duplicates. The molecular identification of the strain MP24 based on the ribosomal DNA internal transcribed spacer (ITS) sequence analysis was applied for phylogeny studies. Glass bead beating, followed by phenol–chloroform extractions, was used for the extraction of genomic DNA from the isolated yeast (Amberg et al. 2005). The forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and the reverse primer ITS4 (5′-TCCTCCGCTTATT GATATGC-3′) were used for the amplification of the PCR amplicons corresponding to the ITS1–5.8S–ITS2 rDNA regions (White et al. 1990). The PCR reactions were carried out on a thermal cycler (BioRad) with an initial

denaturation at 95 °C for 5 min and then amplification was performed by 35 cycles of 94 °C for 1 min, 54 °C for 30 s, and 72 °C for 1 min. The final extension was for 10 min at 72 °C. The PCR product was purified using the PCR purification kit (Fermentas, Germany) and sequenced in both directions on the automated DNA sequencer (Macrogen Company, Seoul, Korea). The results from DNA sequencing were compared with other close yeast strains from the GenBank database using the BLASTN algorithm. The ClustalX programme was used for the multiple alignments of the nucleotide sequences. The phylogenetic tree was created using the neighbour-joining method based on Kimura's two-parameter model with 1000 bootstrap replicates in the MEGA software (Version 6.0) (Tamura et al. 2013).

Bioconversion by a resting culture

For preparation of the resting cell as a biocatalyst for the synthesis of vanillin from isoeugenol, the strain MP24 grew (50 ml in 250-ml flask at 28 °C, 200 rpm) on YPD broth media supplemented with 1 g/l of isoeugenol till the end of the exponential growth phase. The collected biomass (centrifugation $10,000 \times g$ for 10 min at 4 °C) was washed three times with the saline phosphate buffer [K_2HPO_4/KH_2PO_4 (100 mM), pH 5.8] before being re-suspended in the same buffer media. The bioconversion reaction was carried out in a 250-ml Erlenmeyer flask containing 50 ml of the saline phosphate buffer supplemented with 5 g/l of isoeugenol, 2% (v/v) DMF, and biomass concentrations as wet weight of cells ranging from 2.5 to 12.5 g/l. The reaction mixture was incubated for 24 h on a rotary shaker (200 rpm) at 28 °C. The decrease in the isoeugenol substrate concentration and the increase in vanillin and vanillic acid products in the bioconversion media were analysed by UV–Vis spectrometry, TLC, and HPLC. Controls were made with uninoculated same media containing 5 g/l of isoeugenol. All the experiments were performed in triplicates.

Analytical methods

The TLC analysis was done on pre-coated silica gel 60F254 (Merck, Germany) for the identification of bioconverted metabolites from isoeugenol. The samples were loaded on the TLC plates and developed in the solvent system containing *n*-hexane and ethyl acetate (3:4 v/v). After development, the plate was removed, air dried, and visualized under a UV transilluminator (TS-36, USA) at 254 nm. The formation of vanillin and vanillic acid products was also characterized by UV–Vis spectroscopy using Analytik Jena's spectrophotometer SPECORD 210. Quantitative detection of the bioconversion products of isoeugenol was

performed by a crystal 200 series HPLC system (ATI Unicam, Cambridge, UK) using a Perfectsil target C18 column (150×4.6 mm, 5 μ). The mobile phase consisted of 10% (v/v) acetonitrile in 0.1% (v/v) formic acid (Solvent A) and 35% (v/v) acetonitrile in 0.1% (v/v) aqueous formic acid solution (Solvent B) with 30-min gradient conditions at a flow rate of 1 ml/min with the UV detector set at 270 nm (Ashengroph et al. 2011). Components were identified by comparison of retention times and peak areas with those of pure standards. The retention times of solutes were as follows: isoeugenol, 23.1 min; vanillin, 8.8 min and vanillic acid, 5.4 min. The produced vanillin with *T. asahii* strain MP24 resting cells was scraped out from TLC plate, extracted by chloroform and concentrated by evaporation. The chemical structure of the purified vanillin was confirmed by mass spectrometry and X-ray diffraction (XRD). Mass spectra were recorded by an electron impact at 70 eV on a Micromass platform spectrometer (Manchester, UK). An XRD was conducted with the Bruker D8 Advanced model with CuK α radiation (1.54 Å).

Results and discussion

Screening of isoeugenol bioconverting culture

In this study, a bioconversion process was developed employing yeast whole-cell catalysts for the production of high-value products, such as vanillin and vanillic acid from isoeugenol. A primary screening method based on the enrichment technique was tested for the isolation of isoeugenol-tolerant yeasts on the YPD plates supplemented with 5 g/l of isoeugenol. Based on morphology, 40 isoeugenol-tolerant cultures were isolated from the rhizosphere of the collected aromatic plants in different regions of Iran and were further tested based on the ability to degrade isoeugenol as the sole source of carbon and energy in the mineral media, as described in the “[Materials and methods](#)” section. Among the cultures obtained, nine isolates were confirmed which could utilize isoeugenol as the sole carbon and energy source during the growth on the isoeugenol agar medium. To examine the potential of the isolates to bioconvert isoeugenol into vanillin under growing cells conditions, a two-step bioconversion process was used (see the “[Materials and methods](#)” section). Figure 2 shows the molar conversion efficiency of isoeugenol into vanillin (mole vanillin accumulated/mole isoeugenol supplemented) using the conversion of yeast strains. Isolate MP24 was the most potent strain converting 18.6% of isoeugenol into vanillin. Molar yield conversions of the other isoeugenol-converting isolates were between 6.4 and 15.5%. Based on the results, the strain MP24 isolated from the rhizosphere of *Ocimum basilicum* L., showing higher

Fig. 2 Molar conversion efficiency of isoeugenol into vanillin using growing cultures of the isoeugenol-degrading yeast isolates. Cultures initially grown in YPD broth media for 24 h and then isoeugenol substrate was fed with initial concentration of 5 g/l in the presence of 2% (v/v) DMF. Bioconversion reactions were performed at 28 °C in a rotary shaker (200 rpm) for 24 h. Vertical bars show standard deviation ($\pm$ SD) among three replicates

vanillin production compared to other isolates, was selected for further work.

Characterization and identification of the strain MP24

Initial characterization of the selected strain MP24 was performed by morphological and biochemical features. The morphology of the strain on the YPD agar after 48 h of incubation at 28 °C is shown in Fig. 3. The vegetative cells are ovate, long-ovate in terms of their appearance and are found as single cells, in pairs or in clumps. Budding yeast cells, arthroconidia, blastoconidia, and pseudomycelium were produced. The details of growth reactions and other biochemical characteristics of the strain MP24 are given in Table 2. According to these preliminary tests, isolated strain was tentatively characterized as *T. asahii*. For further confirmation, a phylogenetic analysis, based on the amplification of ITS1-5.8S-ITS2 rDNA region sequences, was carried out. The phylogenetic position of the strain MP24 in relation to close species of this genus is shown in Fig. 4. From the results, the strain was identified as a newly isolated strain of the genus *Trichosporon*, which is phylogenetically close to *T. asahii* (Gene Bank accession number

KT439071). *Trichosporon* spp. are anamorphic basidiomycetous yeasts that are extensively distributed in nature; these have been isolated from air, cheese, lake water, seawater, soil, wastewater, wood pulp, and clinical specimens (Colombo et al. 2011). The biotechnological interest in the use of *Trichosporon* spp. is primarily drawn from their ability to degrade various aromatic compounds and xenobiotics in biodegradation and bioremediation processes (Bergauer et al. 2005). Recently, some species of *Trichosporon* have been developed for the biodecaffeination process (Lakshmi and Nilanjana 2011), production of bioemulsifiers (Monteiro et al. 2012), and biotransformation of alicyclic hydrocarbons (Dallinger et al. 2016). However, to our knowledge, no investigation has previously been done on the synthesis of vanillin by the *Trichosporon* genus.

Bioconversion of isoeugenol by resting cell of the strain MP24

In further bioconversion experiments, the resting cell strategy was examined for the bioconversion of isoeugenol into vanillin. In this strategy, biomass duplication was inhibited (Ashengroph et al. 2011, 2012). Cells in the YPD broth media supplemented with isoeugenol (1 g/l, as an inducer) were harvested at the end of the exponential growth phase by a refrigerated centrifuge and washed; the final cells were re-suspended in 100 mM of $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 5.8) and then incubated at 28 °C with shaking at 200 rpm in the presence of 5 g/l of isoeugenol and 2% (v/v) DMF. The correlation between the formation of vanillin and the initial biomass concentration (wet weight) in the conversion reaction was determined (Fig. 5). As seen in Fig. 5, a maximum yield of vanillin accumulation (40.5%) was achieved with 7.5 g/l of wet biomass after 24 h of incubation. Further increase in the biomass concentration resulted in a decrease in the yield of vanillin. It can be implied that the high biomass density may result in insufficient oxygen transfers across biomass and/or fast substrate depletion (Abdelkafi et al. 2006). The depletion of isoeugenol and the possibility of vanillin and vanillic acid accumulation in the reaction mixture amended with 7.5 g/l resting cells of *Trichosporon asahii* MP24 as a

Fig. 3 Morphology of *T. asahii* MP24. **a** Vegetative cells, **b** arthroconidia and blastoconidia, **c** pseudomycelium of formed on YPD agar after 48 h at 28 °C. Bars, 10 μm

Table 2 Physiological and biochemical characteristics of *T. asahii* MP24

Tests	Strain MP24
Sucrose	+
Glucose	+
Inulin	—
Raffinose	—
Galactose	+
Lactose	+
Trehalose	+
Maltose	+
Salicin	+
Xylose	+
D-Arabinose	+
Manitol	—
Glycerol	—
D-Glucosamine	+
Myo-Inositol	—
Citrate	+
Cellobiose	+
KNO ₃	+
L-Lysine	+
Nitrate	—
Nitrite	—
Cycloheximide 0.01%	+
Cycloheximide 0.1%	+
Urease	+
Starch formation	—
Growth in 25–35 °C	+
Growth in 37 °C	+

Fig. 4 Phylogenetic tree of strain MP24 and close species in the Trichosporonales based on the ITS1–5.8S–ITS2 regions of rDNA sequences. The numbers on branches represent the confidence level from 1000 replicate bootstrap sampling that supported the internal branches by 50% or higher. *Cryptococcus podzolicus* was used as the outgroup. Bar, 0.02 substitutions per nucleotide position. GenBank accession numbers are given in parentheses **Fig. 5** The effect of different concentrations of biomass (wet weight) on the yield of vanillin in the potassium phosphate buffer (100 mM, pH 5.8) of *T. asahii* strain MP24 supplemented with 5 g/l isoeugenol as the sole carbon and energy source in the presence of 2% (v/v) DMF at 28 °C, after 24 h of incubation and agitation 200 rpm. The vertical bars show standard deviation ($\pm$ SD) among three replicates

biocatalyst and 5 g/l of isoeugenol substrate were monitored by UV–Vis spectrometry, TLC, and HPLC analysis (Fig. 6). The UV spectra results demonstrate hypsochromic shifts in the peak maxima (Fig. 6a). The conversion of isoeugenol ($\lambda_{\max}$ 206 and 258 nm) to metabolites with absorption maxima ($\lambda_{\max}$ 277, 318, and 347 nm) could contribute to the accumulation of vanillin ($\lambda_{\max}$ 277, 318, and 347 nm) and vanillic acid ($\lambda_{\max}$ 247 and 288 nm) in the reaction mixture under resting cell incubation. The thin layer chromatography profile of the bioconversion process revealed two distinct peaks at the retention factor (R_f) of

Fig. 6 UV spectral shift (a), TLC (b) and HPLC (c) chromatograms showing potential isoeugenol-converting products in the reaction mixture with resting cells of *T. asahii* strain MP24. The reaction mixture contained potassium phosphate buffer (100 mM, pH 5.8),

5 g/l isoeugenol, 2% (v/v) DMF and 7.5 g/l (wet weight) of biomass. The reaction was performed (200 rpm) at 28 °C for 32 h. (A standard vanillin; B standard vanillic acid; C standard isoeugenol; D reaction mixture)

0.75 and 0.36, which were identical to that of authentic standards of vanillin and vanillic acid, respectively (Fig. 6b). The HPLC chromatograms also confirmed a decrease in the amount of the isoeugenol substrate and the formation of two major metabolites of vanillic acid (retention time or R_t of 5.4 min) and vanillin (8.8 min) in the reaction mixture (Fig. 6c). No biotransformed products of isoeugenol have been detected in control experiments (uninoculated). This may be due to the fact that isoeugenol was not partially transformed abiotically in the reaction mixture. The time span of isoeugenol degradation and formation of bioconverted products in the reaction mixture containing the potassium phosphate buffer (100 mM, pH 5.8), 7.5 g/l of resting cell of *T. asahii* MP24, and 5 g/l isoeugenol in the presence of 2% (v/v) DMF is shown in Fig. 7. There was a significant increase in the amount of vanillin production for up to 32 h. After 32 h of the initiation of conversion time, the highest concentration of vanillin (2.4 g/l), generated by the resting cells of *T. asahii* MP24, resulted in a molar yield of 52.5% and then declined to its minimum value of 1 g/l at the end of the conversion process. The increase in the incubation period was deleterious to the formation of vanillin due to the oxidation activity that turns vanillin into vanillic acid by the resting cells of the strain MP24. The vanillic acid level continued to increase to its maximum of 1.8 g/l (molar yield 35.8%) for 48 h after incubation and could not be improved with increased incubation time owing to further metabolizing of vanillic acid by the strain MP24. The vanillic acid concentration reached its maximum value (1.8 g/l) after 48 h of incubation, corresponding to the isoeugenol conversion

Fig. 7 The effect of incubation time on the biological conversion of isoeugenol into vanillin and vanillic acid with resting cells of *T. asahii* strain MP24. The reactions were done at 28 °C for 56 h with shaking at 200 rpm in 250-ml Erlenmeyer flasks containing 50 ml of potassium phosphate buffer (100 mM, pH 5.8) supplemented with 7.5 g/l of resting cell as wet weight, 5 g/l of isoeugenol and in the presence of 2% (v/v) DMF. The vertical bars show standard deviation ($\pm$ SD) among three replicates

yield of 35.8%. The total concentration of vanillin and vanillic acid obtained from this biotransformation process was 4.2 g/l (total molar yield of 88.3%). The degradation of isoeugenol was also examined, showing that almost all the substrates (5 g/l) were consumed after 56 h of incubation. The purified product was confirmed by an interpretation of the standard data obtained from XRD and mass spectroscopy, and comparison with authentic vanillin as a

Fig. 8 Mass Spectroscopy analysis (a) and X-ray diffraction patterns (b) of purified vanillin prepared in the bioconversion reaction by resting cells of *T. asahii* MP24

reference. The mass spectroscopy analysis of the bio-synthesized vanillin showed a distinct mass peak of m/z 151.9, which corresponds to the molecular weight of vanillin (Fig. 8a). As can be observed in Fig. 8b, X-ray diffraction patterns of purified vanillin exhibited the same diffraction peaks as that of a reference vanillin that can be indexed to the crystallographic structure of prepared vanillin. Many different bacterial strains, especially *Pseudomonas* and *Bacillus* species, have so far been isolated and used for studying the bioconversion of isoeugenol into vanillin under different operating conditions (Table 1). Using the resting cells of *P. putida* IE27, 16.1 g/l vanillin was obtained with 71% molar yield after 24 h incubation in the reaction mixture supplemented with 150 mM isoeugenol in the presence of 10% (v/v) dimethylsulfoxide (DMSO), under optimized reaction conditions. This is the highest vanillin molar yield from isoeugenol by non-engineered strains. In spite of the high molar efficiency of the above reaction, due to utilization of non-GRAS microorganism as well as the use of polar aprotic solvent (10% DMSO) with high polarity and high boiling point, which completely makes it almost impossible to isolate it from vanillin produced in the reaction mixture, it is impossible or difficult to use the bioconversion process on an industrial scale to produce natural vanillin.

Conclusion

Until now, several reports have described the isolation of the different groups of isoeugenol bioconverting bacteria with the capability production of vanillin. However, only few studies have investigated the conversion of isoeugenol into vanillin using fungi (Abraham et al. 1988; Ashengroph et al. 2011) and none of the reports has used the *Trichosporon* genus. We have described, for the first time, a newly isolated yeast strain of the *T. asahii* that may be considered an efficient biocatalyst for the bioconversion of isoeugenol into vanillin. The maximum yield of vanillin was 52.5% at 28 °C and the agitation speed of 200 rpm in

the potassium phosphate buffer (100 mM, pH 5.8) with isoeugenol concentration of 5 g/l after 32 h of incubation by the resting cell with a concentration of 7.5 g/l. Further investigations on the optimization process and the molecular characterization of the enzymes involved in the bio-transformation process may facilitate the large-scale production of biovanillin from isoeugenol.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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