Process Biochemistry 122 (2022) 86–94

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Whole-cell biocatalyzed organic solvent-free conversion of dill oil to *cis*-(-)-dihydrocarvone rich aromatic hydrosol: Chemical and aroma profiling

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ARTICLE INFO

Keywords:
Biotransformation
Whole-cell biocatalyst
Essential oil
Sensory analysis
Fusarium

ABSTRACT

Biotechnologically produced flavours and fragrances are labeled as 'natural' and in great demand. In the current study, *Fusarium equiseti* was utilized as the whole-cell biocatalyst for the selective transformation of S-(+)-carvone in the dill oil to *cis*-(-)-dihydrocarvone [*cis*-(-)-DHC]. In an optimized condition (pH 5–6, dill oil 1.0 g/L and incubation period 24 h) *F. equiseti* produced S-(+)-carvone metabolites, dominated by *cis*-(-)-DHC (92–96% through gas chromatography-mass spectrometry or GC-MS) in nearly quantitative conversion (~99%). In the next step, distillation of the whole culture isolated the product in the form of *cis*-(-)-DHC rich (0.33 g/L) hydrosol; thus eliminating the use of organic solvent for the extraction and reducing the solvent soluble off-odour in the product. Detailed sensory analysis demonstrated the uniqueness of hydrosol aroma in reference to the dill oil and characterized it as sweet, minty, fresh, spicy and herbal. Headspace-GC-MS analysis supported by the odour activity value and sensory profile showed *cis*-(-)-DHC as the key odorant in the produced hydrosol. The developed bioconversion technique was novel in terms of its high selectivity towards *cis*-(-)-DHC and organic solvent-free downstream processing, producing a unique aromatic hydrosol.

1. Introduction

Dihydrocarvone (DHC), a cyclic oxygenated monoterpene possessing four stereoisomers is a useful chiral building block in organic synthesis [1]. For example, *trans*-(+)-dihydrocarvone has been used widely as the starting precursor for the total synthesis of many sesqui and diterpenes and their analogues (*e.g.* polygodial, thapsigargin, chrysanthemol, decipienin A, α-agarofuran, bakkenolide III, glutinone, tubingensin A, pleuromutilin) [2–10]. The use of *cis*-dihydrocarvone for the chiral synthesis of rotundone and carvolactone is known [11,12]. It is also important in flavour and fragrance due to its carvone/menthone like aroma [13,14]. However, natural access to its optically pure isomers is difficult due to the low abundance in plant sources like caraway, dill seed *etc* and challenging purification [15–17]. Alternatively, bioflavours can be produced through enzymatic (whole-cell/cell-free) methods. Whole-cell biocatalysis plays a pivotal role in biotechnological production of flavours in which metabolically active cells act as the stock of enzymes and cofactors, making it an economic and scalable technique [18–20]. It can be a useful means for the development of economically viable processes converting commercially available essential oils to new bioflavours or aroma. In this study *Fusarium equiseti*, a fungal plant pathogen was found to be an efficient whole-cell biocatalyst for the selective conversion of S-(+)-carvone in dill (*Anethum graveolens*) oil to *cis*-(-)-DHC. Though there are several reports on the microbial transformation of S-(+)-carvone to DHCs, they have one or more drawbacks including (i) lack of selectivity in *cis* versus *trans*-DHC and (ii) further reduction of DHCs to alcohols like carveols/dihydrocarveols; thus reducing the selectivity and purity of the desired DHC isomer [21–26]. Therefore, a microbial technique that can selectively produce *cis*-(-)-DHC with high substrate conversion may have commercial as well as scientific significance.

The use of safer solvents is one of the twelve key principles of green

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https://doi.org/10.1016/j.procbio.2022.08.019
Received 17 May 2022; Received in revised form 26 July 2022; Accepted 13 August 2022
Available online 17 August 2022

chemistry [27]. During the downstream processing of a whole-cell biocatalyzed process, the isolation of organic metabolites generally requires the extraction of fermentation broth with organic solvents such as ethyl acetate, dichloromethane, chloroform or hexane. To eliminate the use of organic solvent, distillation was incorporated into the currently developed technique to extract the produced metabolites in the form of *cis*-(-)-DHC rich hydrosol. It may also reduce the possibility of organic solvent-soluble mycotoxin contaminants (such as zearalenone, trichothecens and fumonisins produced by toxigenic *Fusarium* species including *F. equiseti*) [28] or non-volatile off-flavours in the final product. Furthermore, detailed sensory analysis of the biocatalytically produced hydrosol characterized the aroma, indicating its relevance in food and flavour.

2. Materials and methods

2.1. Chemicals

Technical grade solvents were distilled before use for the extraction and chromatography experiments. Deionized water was collected from the Milli-Q water purification system for the preparation of culture media, buffers and sensory analyses. Deuterated chloroform (99.8 atom %D), eugenol (99%) and 1,4-dimethoxybenzene (99%) were procured from Sigma-Aldrich, St. Louis, MO, USA. Silica gel (60–120 mesh), sodium sulfate and buffer chemicals were purchased from Avra Synthesis, Hyderabad, India. HPLC grade solvents, potato dextrose agar and broth were procured from Merck, Darmstadt, Germany. Dill (*A. graveolens*) oil of therapeutic grade was obtained from Deve Herbes, New Delhi, India.

2.2. General procedures

Thin layer chromatography (TLC) was performed on silica gel G precoated (250 μm) aluminium plates with 1:1 *v/v* dichloromethane and *n*-hexane mixture as the mobile phase. Anisaldehyde based staining agent was used for the spot visualization on the TLC plate. The optical rotation was measured in a polarimeter made by Anton Paar (MCP 200).

2.3. Gas chromatography-mass spectrometry (GC-MS)

The gas chromatography-mass spectrometry (GC-MS) analysis was performed in Agilent 8890 gas chromatograph coupled with Agilent 7010B triple quadrupole mass spectrometer. Analytes were separated using HP-5MS UI capillary column (30 m × 0.25 mm × 0.25 μm). Helium with a flow rate of 1.0 mL/min was employed as the carrier gas. The injector temperature was set at 250 °C whereas the initial column temperature was kept at 50 °C for 1.0 min. Then, a temperature gradient of 8 °C/min was set till 200 °C and held for 2.0 min, followed by a ramp of 15 °C/min up to 260 °C and held for 3 min (total run time 28.75 min). GC-MS samples were prepared in HPLC grade ethyl acetate (~150 ppm) for the liquid injection and 0.3 μL of the sample solution was injected (split ratio 10:1) using PAL3 RSI 85 autosampler. The relative percent of the individual analytes was determined as area under the peak. For the data processing and the structural prediction, Agilent MassHunter Qualitative Analysis 10.0 software and integrated NIST 2017 mass spectral library were used respectively. Also, linear retention index (RI) values relative to C_8-C_{20} *n*-alkanes were calculated for the identified peaks.

For the headspace (HS) analysis, an integrated PAL3 RSI 85 headspace autosampler was employed. About 0.2 μL oil or 300.0 μL hydrosol was taken in a 20 mL screw-top headspace glass vial fitted with a septate (PTFE and silicone) magnetic cap. The capped vial containing the sample was agitated at 40 °C with a speed of 250 rpm for 3.0 min (5.0 s on and 2.0 s off). The syringe temperature was kept at 50 °C and 0.05 mL headspace sample was injected in GC-MS under the same condition and temperature program as that of liquid injection.

2.4. Nuclear magnetic resonance (NMR) spectroscopy

A 400YH FT NMR spectrometer (400 MHz, Jeol) was used to record ^{1}H and ^{13}C NMR spectra. Deuterated chloroform (600 μL) was used for the preparation of the NMR samples. MestReNova 14.0.0 software was used for the data analysis. Chemical shift (δ) values were in ppm and the residual solvent peak of deuterated chloroform (δ_H 7.26 ppm) was used as the reference in the proton NMR spectra.

2.5. Purification of S-(+)-carvone

S-(+)-carvone (0.8 g) was purified from dill oil (2.0 g) through silica gel column (H×D 23 ×2 cm) chromatography using an increasing percentage of ethyl acetate (0–30% *v/v*) in *n*-hexane as the mobile phase. The identity and purity (>99%) of S-(+)-carvone were confirmed through GC-MS, NMR and specific rotation analyses.

2.6. Identification, maintenance and culturing of F. equiseti

F. equiseti was sub-cultured on potato dextrose agar (PDA) slant at 30 °C for 3 days and stored at 4 °C. It was isolated from a contaminated slant of mushroom on PDA in our laboratory. The identity of this fungal culture was confirmed through the sequence analysis of internal transcribed spacer (ITS4 and ITS5). It was deposited to the National Fungal Culture Collection of India (NFCCI), Pune, India with an accession number NFCCI 5271. The inoculum (~5% *v/v*, 0.5–1.5 ×10^7 spores/mL), prepared from a thickly grown fresh culture on PDA slant (H×W 9.0–10.5 ×1.5–1.7 cm) was used for the inoculation of potato dextrose broth (PDB) (50 mL×3, pH 5.1 ± 0.2) in 250 mL *Erlenmeyer* flasks under aseptic condition. The flasks were incubated at 28 °C and 150 rpm in an orbital shaking incubator (shaking diameter 2.5 cm) which was used throughout the study.

2.7. Analytical scale biotransformation

In a 3-days well-grown culture (packed cell volume 0.60–1.25%), 50.0 mg dill oil (substrate concentration 1.0 g/L) in 300 μL acetone was added and incubated at 28 °C and 150 rpm for 24 h. The fungal biomass was removed from the broth through a muslin cloth and extracted using ethyl acetate (25 mL×2). The biomass was extracted separately with ethyl acetate which was combined with the broth extract. The organic layer was evaporated at 42 °C under reduced pressure (150–200 mbar) to obtain the crude metabolite which was further analyzed through GC-MS. Besides, two control experiments were set up; (i) substrate control: dill oil in PDB without organism and (ii) organism control: *F. equiseti* in PDB without dill oil.

2.8. Study on the evaporation of limonene

D-Limonene (25.0 mg) was added into 250 mL *Erlenmeyer* flask containing 50 mL sterile distilled water and incubated at 28 °C and 150 rpm for 24 h and finally extracted with ethyl acetate (25 mL × 2). The organic layer was concentrated at 42 °C under reduced pressure (150–200 mbar). The recovery of limonene was recorded and the purity was checked through GC-MS analysis. In another set of experiment, D-limonene (26.0 mg) was individually taken in three different 5 mL glass vials. The first vial was screw-capped (close system); the second one was plugged with cotton and the third vial was kept open. All the vials were simultaneously incubated at 28 °C in static condition for 24 h and finally, the weight of limonene was recorded. The purity of limonene after the incubation was also tested through GC-MS.

2.9. Resting cell experiment

The well-grown culture of *F. equiseti* was filtered through a muslin cloth to obtain the mycelium that was washed with sterile deionized

water 3–5 times to wash off the residual broth. Then, the mycelium was squeezed gently to remove the water yielding 0.40–0.65 g wet biomass. It was taken into 50 mL Mcllvaine buffer of pH 5.0, added with dextrose (300 mg) and dill oil (10.0 mg in 50 μL acetone) in a 250 mL flask. It was incubated at 28 °C and 150 rpm for 24 h. Finally, the broth and biomass were extracted, concentrated and analyzed in GC-MS.

2.10. pH Optimization study

The resting cell experiment was set up in 6 flasks each containing 50 mL Mcllvaine buffer of different pH (3, 4, 5, 6, 7 and 8). The broth and biomass in the individual flask were extracted, and analyzed similarly in GC-MS.

2.11. Effect of the inoculum size

The inoculum (0.8–1.3 $\times 10^7$ spores/mL) was prepared by gently rubbing the surface (with a sterile loop) of a thickly grown fresh slant (H×W 9.0–10.5 ×1.5–1.7 cm) of *F. equiseti* added with 8.0 mL sterile water. The inoculum of varying volume (0.25%, 1.0%, 2.5%, 5.0% and 7.5% *v/v*) was added to 50 mL PDB and incubated at 28 °C and 150 rpm for 3 days. The biomass concentration in these cultures was represented through packed cell volume or PCV [29]. For the estimation of PCV, 50 mL culture was filtered through the muslin cloth to obtain the fungal biomass. It was washed repeatedly with water and finally squeezed to obtain the biomass cake which was taken in a graduated micro-centrifuge tube and centrifuged for better packing. The biomass volume was recorded and the PCV was expressed in % *v/v*. In another set of cultures grown with varying inoculum sizes, S-(+)-carvone (0.5 g/L) was added and incubated in the same condition for 12 h. The extraction and analysis of % transformation were performed similarly.

2.12. Time-course experiment

For studying the time-dependent progress of the bioconversion, dill oil at different concentrations (1.0, 2.0 and 3.0 g/L) was added individually in 3-days well-grown culture of *F. equiseti* (packed cell volume 0.60–1.25%) in 50 mL PDB per flask (250 mL). One mL aliquot was taken out aseptically from each flask after 11, 24, 48 and 72 h and extracted with 1.0 mL ethyl acetate. The ethyl acetate layer was passed through anhydrous sodium sulfate and analyzed through GC-MS to check the progress of the conversion. The time-course experiment for S-(+)-carvone was also carried out similarly with 0.5, 0.7, 1.0 and 1.5 g/L concentrations. The % conversion of S-(+)-carvone was calculated using the formula: [100 - residual % of S-(+)-carvone after fermentation].

2.13. Preparative scale transformation and the preparation of hydrosol and hydrosol-extract

For the preparative scale conversion, 12 flasks (250 mL) each containing well-grown culture of *F. equiseti* in 50 mL PDB (packed cell volume 0.60–1.25%) was added with 50.0 mg dill oil in 300 μL acetone (in total 600.0 mg oil, 1.0 g/L) and incubated at 28 °C and 150 rpm for 24 h. Then, the broth and biomass from 12 flasks were pooled together in a 2 L Clevenger-type apparatus and the hydrosol (525 mL) was distilled out. The distillation apparatus was operated in continuous collection mode without recirculation of the water. The produced hydrosol was stored at 4 °C in dark. To prepare the extract, 325 mL hydrosol was extracted with ethyl acetate (300 mL×3) and the combined organic layers were concentrated at 42 °C under reduced pressure (150–200 mbar) to obtain 155.0 mg colourless oil (*i.e.* hydrosol-extract).

2.14. Preparation of the solvent-extract and characterization of cis-(-)-DHC

Preparative scale transformation was carried out similarly with 11 flasks of *F. equiseti* culture containing in total 550.0 mg of dill oil (1.0 g/L). The culture broth from all the flasks was pooled together after the separation of biomass through a muslin cloth and extracted with ethyl acetate (250 mL×2). The biomass was also extracted separately and mixed with the broth extract. Ethyl acetate was removed similarly to obtain 220.0 mg solvent extract. This extract was loaded on a silica gel column (60–120 mesh, L×ID 18 ×1.1 cm) and eluted with 300 mL 1.5% ethyl acetate in *n*-hexane (*v/v*). The fractions showing pure *cis*-(-)-DHC on the TLC plate were pooled and concentrated. It yielded 106.2 mg *cis*-(-)-DHC which was stored at 4 °C in dark. The purified product was structurally characterized through NMR (^{1}H, ^{13}C) spectroscopic and mass spectrometric (GC-MS) data and specific rotation value.

2.15. Quantification of cis-(-)-DHC in the hydrosol extract and hydrosol

(a) GC-MS technique: To quantify *cis*-(-)-DHC in the hydrosol extract through GC-MS, eugenol (99%) was used as the internal standard (IS). The amount of *cis*-(-)-DHC in the assay was determined using the formula: [(peak area ratio of *cis*-(-)-DHC versus IS) × added amount of IS / relative response factor]; relative response factor = response factor ratio of *cis*-(-)-DHC versus IS. The ratio of peak area versus concentration of an analyte is referred to as its response factor. The assay sample contained 0.105 mg hydrosol extract and 0.102 mg IS in 1.0 mL ethyl acetate and 1.5 μL of the sample was injected. (b) ^{1}H qNMR technique: 1,4-Dimethoxybenzene (99%) was used as the IS in ^{1}H qNMR based quantification. The assay sample contained 5.8 mg hydrosol extract and 0.53 mg IS in 700 μL deuterated chloroform. The amount of *cis*-(-)-DHC in the assay was calculated using the formula: $M_{DHC} = M_{IS} \times PA_{DHC}/PA_{IS}$ [abbreviation: M_{DHC} = moles of *cis*-(-)-DHC, M_{IS} = moles of IS, PA_{DHC} = peak area of *cis*-(-)-DHC at δ_H 4.82 ppm (br s, 1 H) and PA_{IS} = peak area of IS at δ_H 3.77 ppm (s, 6 H)]. The quantity of *cis*-(-)-DHC in the hydrosol extract was further used to calculate its amount in the produced hydrosol.

2.16. Difference from control (DFC) test

The difference from control (DFC) test was performed to assess the odour difference in hydrosol, hydrosol extract, solvent extract and *cis*-(-)-DHC in reference to the dill oil (control) [30]. Again, dill oil was taken as the blind control. The samples (0.5% *v/v*) were prepared in 0.5% methanol in water, coded and presented with the smelling strips. In total eight panelists including six male and two female adults with no nasal disorder (age 25–35 years) were involved in the test. They were instructed to assess and score the odour difference for the individual samples on a five-point "difference scale" (0 no difference, +1 slightly different, +2 moderately different, +3 largely different and +4 no similarity) relative to the control. The test was performed in duplicate by each panelist. The average values of the difference scores were plotted graphically.

2.17. Odour threshold and odour activity value determination

The odour threshold value (OTV) of dill oil, hydrosol, hydrosol extract and *cis*-(-)-DHC was determined through single ascending method [31]. Six dilutions (0.5%, 0.1%, 0.05%, 0.01%, 0.005% and 0.0005%) were prepared for the dill oil, hydrosol extract and *cis*-(-)-DHC in water containing 0.5% methanol. In the case of hydrosol, four dilutions (10.0%, 1.0%, 0.1% and 0.01%) were made in water. The same group of panelists was instructed to detect the odour of the individual samples through smelling strips in the order of increasing concentration. OTV was recorded as the lowest concentration at which odour stimulus was detected. Each test was performed in duplicate by the panelists. The average of the OTV values was calculated and also its distribution among the panelists was plotted. The odour activity value (OAV) of an odorant was calculated by dividing its concentration by OTV.

2.18. Triangle test

Triangle test was performed to confirm the perceivable sensory (odour) difference between dill oil and hydrosol [32]. The sample of dill oil (0.5% *v/v*) was prepared in 0.5% methanol in water, coded and provided with the smelling strips. The coded hydrosol was taken without any dilution. The samples were assessed by sixteen panelists (11 males and 5 females, age 25–35 years). Each panelist was presented with three coded samples, two of which were the same (dill oil) and the third one was the hydrosol. The assessors were instructed to identify the odd one and the correctness of their response was recorded. The difference was concluded at 5% significance level on the basis of the number of correct responses. Another set of triangle test was also performed between the dill oil and hydrosol extract in which both the samples (0.5% *v/v*) were prepared in water containing 0.5% methanol.

2.19. Descriptive sensory (odour) analysis

The Sensorial comparison among dill oil, hydrosol extract and solvent extract was performed through a descriptive sensory analysis [33,34]. A panel of six healthy adults (five males and one female, age 25–35 years), experienced working with flavors and fragrances in the laboratory were selected for this test. Five aroma descriptors (herbal, sweet, minty, fresh and spicy) that are known for the dill oil and DHC were selected. The following samples were used as the descriptor references to train the panelists; (1) herbal: freshly cut grass (*Cynodon dactylon*) and basil leaf, (2) sweet: mandarin oil and honey, (3) minty: peppermint oil, (4) fresh: lavender and eucalyptus oil, (5) spicy: cinnamon and clove oil. The ethyl acetate extract of the culture broth without substrate was also included in the test as the 'culture extract'. The oily samples (5 μL) were taken in a small piece of cotton kept inside a screw-capped glass vial (5 mL). The assessors were instructed to perceive the odour of the individual samples and score each descriptor on a six-point scale (0 = not perceived to 5 = strongly perceived). Each panelist repeated the test at an interval of at least 3 h. The average values of the scores for each descriptor were plotted in a spider web diagram.

3. Results and discussion

3.1. Bioconversion of dill oil and S-(+)-carvone to cis-(-)-DHC rich metabolites by F. equiseti

F. equiseti, a known fungal plant pathogen was isolated as a contaminant in the mushroom cultures on the agar medium. Considering the demonstrated biocatalytic potential of the *Fusarium* species [35], it was further included in our screening program for the sensory alteration of essential oils through whole-cell biocatalysis. Among twenty commercially available essential oils screened with *F. equiseti*, dill oil showed an extensive change in its chemical profile after the fermentation; hence considered for further studies.

D-limonene and S-(+)-carvone are the major constituents of dill oil and their relative abundance were found to be 51.7% and 47.3% respectively (99.0% in total) in the tested oil (Table 1, Fig. 1A) [16,17].

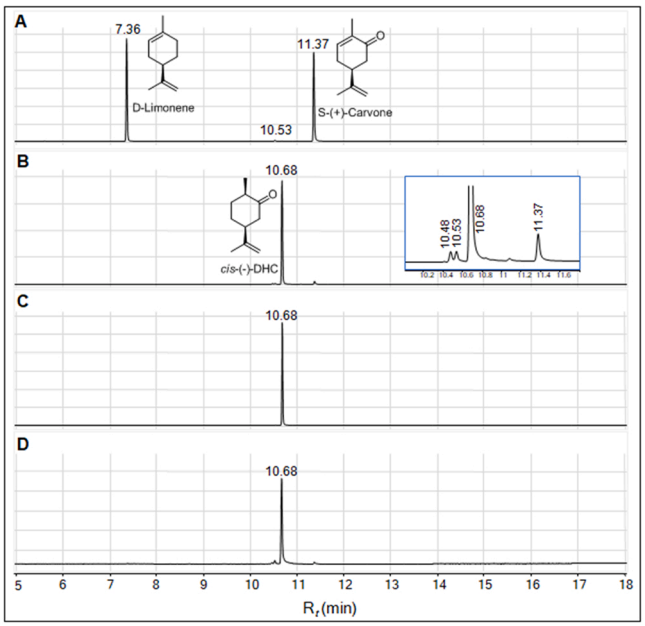

Fig. 1. Stack plot of the GC-MS chromatograms (R_t 5.0–18.0 min) for the (A) dill oil, (B) hydrosol extract [inset is showing the enlarged section in the R_t range 10.0–11.8 min], (C) purified *cis*-(-)-dihydrocarvone (DHC) and (D) headspace analysis of the hydrosol [R_t: retention time].

After the incubation of dill oil (1.0 g/L, 28 °C and 150 rpm) with *F. equiseti* for 24 h, limonene was not detectable and S-(+)-carvone was reduced to 2.5%. Instead, a major metabolite (R_t 10.68 min, 96.2%) was formed which was identified as dihydrocarvone (a hydrogenated analogue of carvone) through its *m/z* value and GC-MS library hit (Table 1, Fig. 1B-C). Silica gel column chromatographic purification followed by GC-MS, NMR (^{1}H and ^{13}C) and optical rotation analyses of this metabolite confirmed its purity (>99%) and identity as *cis*-(-)-dihydrocarvone (DHC). The obtained NMR data [δ_H (ppm): 4.82 (m, 1H), 4.68 (m, 1H), 2.51–2.63 (m, 2H), 2.35–2.44 (m, 2H), 1.81–1.90 (m, 3H), 1.72 (br s, 3H), 1.62–1.65 (m, 1H), 1.08 (d, *J*=7.0 Hz, 3H); δ_C (ppm): 214.1, 146.9, 111.5, 44.6, 44.1, 43.9, 30.6, 26.3, 21.5, 15.6] matched well with the previous reports [36,37]. The specific rotation value of purified *cis*-(-)-DHC was determined as $[\alpha]_D^{25}$ = (-)45.4 (*c* 4.00, $CHCl_3$) which further supported its designated configuration as (2R, 5S). The formation of minor metabolites (relative concentration <1.0%) such as dihydrocarveol was also observed along with *cis*-(-)-DHC (Table 1, Fig. 1B). When purified S-(+)-carvone (0.5 g/L) was incubated with *F. equiseti* in the same condition for 24 h, a very similar metabolite profile, dominated by *cis*-(-)-DHC was observed. It confirmed that S-(+)-carvone (substrate) was metabolized by *F. equiseti* to produce *cis*-(-)-DHC. The resting cell experiment with dill oil and S-(+)-carvone separately, led to the formation of the same metabolites. It further confirmed that the transformation of S-(+)-carvone was mediated by the microbial enzyme(s).

Table 1
GC-MS analysis of the dill oil (used in the current study, liquid injection), hydrosol extract (liquid injection) and hydrosol (headspace) obtained after the incubation of dill oil (1.0 g/L, 28 °C and 24 h) with *F. equiseti* [R_t: retention time, *m/z*: mass to charge ratio, RI_{calc}: calculated retention indices, relative %: peak area sum %, '-': not detected].

R_t (min)	Structural identity	*m/z*	RI_{calc}	Relative %		
				Dill oil	Hydrosol extract	Hydrosol (Headspace analysis)
7.36	D-limonene	136.3 [M^+]	1032	51.66	–	–
10.48	Dihydrocarveol	136.3 [M-H_2O^+]	1199	–	0.69	0.84
10.53	*trans*-(-)-Dihydrocarvone	152.2 [M^+]	1202	0.88	0.63	2.38
10.68	*cis*-(-)-Dihydrocarvone	152.2 [M^+]	1211	0.17	96.19	95.29
11.37	S-(+)-carvone	150.2 [M^+]	1234	47.29	2.49	1.61

On the other hand, the disappearance of limonene after the incubation didn't yield any metabolite. Also, in the substrate control experiment (*i.e.* dill oil in PDB without organism) limonene signal disappeared while S-(+)-carvone underwent no change. It indicated either degradation or evaporation of limonene during the incubation. To confirm, pure limonene (25.0 mg) was incubated in sterile water (50 mL) for 24 h at 28 °C and 150 rpm in a flask (250 mL) which after extraction yielded only 4.6 mg mass (81.6% loss), indicating its evaporation in this simulated experimental condition. In another set of experiments, when pure limonene (25.0 mg) was incubated in 5 mL glass vial at 28 °C in three different conditions (screw-capped, cotton-plugged and open) for 24 h, 45% reduction in weight was observed without any degradation in the open vial. On the other hand, no loss was recorded in the screw-capped condition. These results confirmed the evaporation of limonene from dill oil during the bioconversion process. Adams and co-workers also observed the drastic loss of limonene even after 2 h of incubation with water in a shaking flask (26 °C and 150 rpm) [38]. Thus, *F. equiseti* was found to be an efficient whole-cell biocatalyst for the conversion of S-(+)-carvone in dill essential oil to *cis*-(-)-DHC dominating oily metabolite mixture.

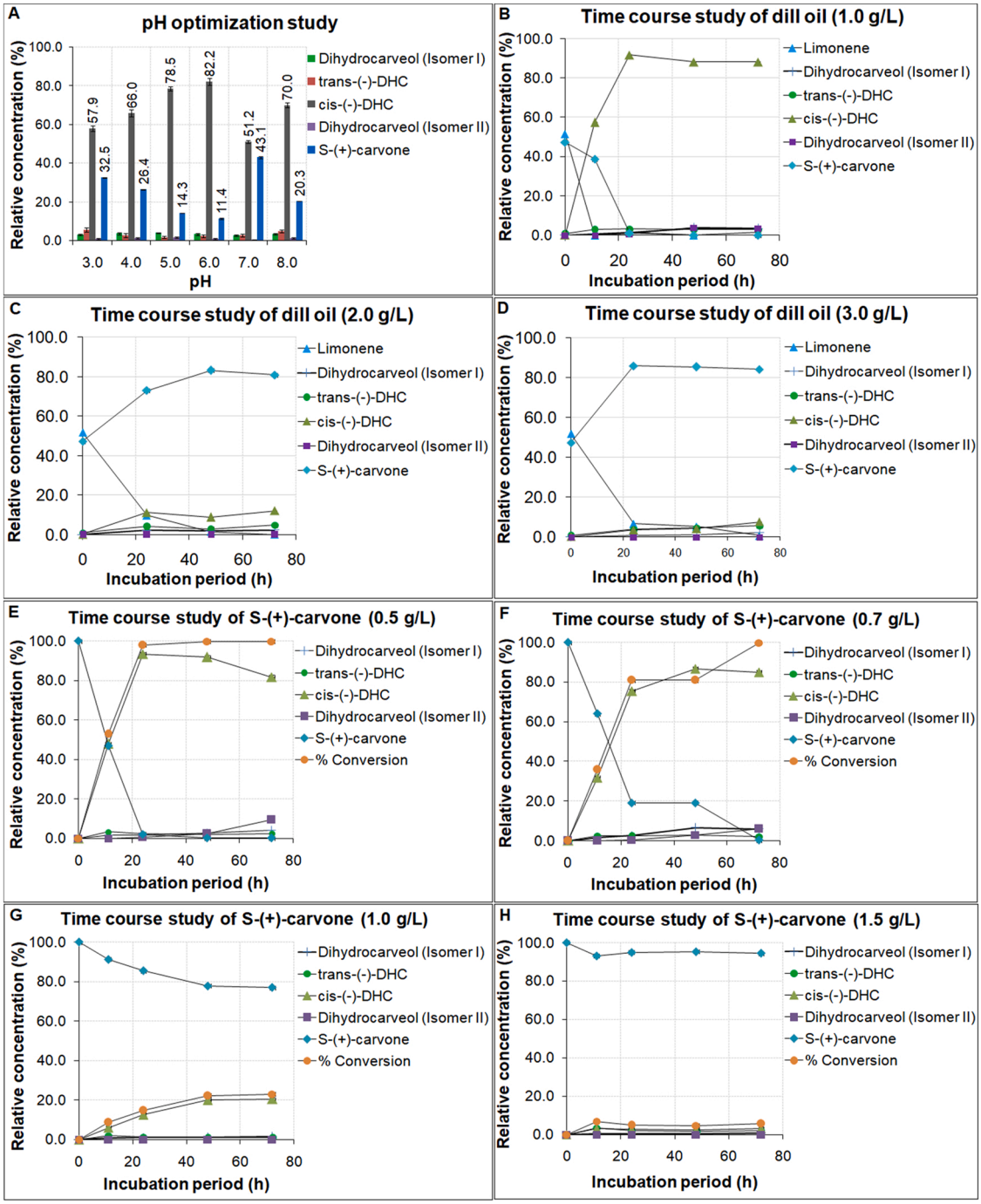

Fig. 2. Optimization studies analyzed through GC-MS: (A) pH (3.0–8.0) optimization study, (B-D) time course study of dill oil up to 72 h in 1.0, 2.0 and 3.0 g/L concentrations, (E-H) time course study of S-(+)-carvone up to 72 h in 0.5, 0.7, 1.0 and 1.5 g/L concentrations.

3.2. Optimization studies: pH, inoculum size, concentration and incubation period

In the next step, critical biotransformation parameters such as pH of the medium, inoculum size, substrate concentration and incubation period were optimized, targeting the quantitative conversion of S-(+)-carvone with high selectivity towards *cis*-(-)-DHC. The pH was optimized through resting cell experiment with dill oil (10.0 mg in 50 mL buffer) performed in the pH range 3–8. The quantitative profile of the crude product varied widely in the tested pH range. It was observed that in the pH range 3–6, the % conversion of S-(+)-carvone increased with increasing pH. The conversion was highest at pH 5 and 6 (85.7% and 88.6% respectively), yielding 78.5% and 82.2% *cis*-(-)-DHC respectively in the extract (Fig. 2**A**). The progress of the transformation slowed down in the pH range 7.0–8.0. Thus, optimized pH was established as 5–6 for the transformation of S-(+)-carvone to *cis*-(-)-DHC by *F. equiseti*. To note, pH of the PDB falls in the optimized range; hence used for the current biotransformation without further adjustment. When the effect of inoculum (0.8–1.3 $\times 10^7$ spores/mL) size was investigated in the range 0.25–7.5% *v/v*, the conversion of S-(+)-carvone (0.5 g/L) didn't vary drastically and remained within 51.5–56.5% after 12 h of incubation (Supplementary material Fig. S1). Although the biomass concentration, represented through its packed cell volume increased gradually from 0.45% to 1.13% with the increasing inoculum size. These results indicated that transformation efficiency was not critically influenced by the inoculum size in the range 0.25–7.5% *v/v* in this process.

Time course study was performed up to an incubation period of 72 h to optimize the substrate concentration and incubation period for the transformation of dill oil with three increasing substrate concentrations (1.0, 2.0 and 3.0 g/L). It was observed that at 1.0 g/L the kinetics was way faster than its higher concentrations. After 24 h, the relative % of *cis*-(-)-DHC in the crude extract reached its highest (91.8%) and the % conversion of S-(+)-carvone was almost quantitative (98.8%) (Fig. 2B). However, the % of *cis*-(-)-DHC slowly decreased (88.3% at 72 h) with progressing time due to its further reduction to alcohols (*i.e.* dihydrocarveol isomers). At 2.0 and 3.0 g/L, the % *cis*-(-)-DHC reached only up to 12.1% and 7.5% respectively after 72 h of incubation (Fig. 2C-D). The slow kinetics was observed possibly due to the substrate and/or product inhibition. Limonene was also left up to 5–10% of the total extract at these high substrate concentrations (2.0–3.0 g/L). To note, the relative concentration of S-(+)-carvone increased in 2.0 and 3.0 g/L kinetic studies (Fig. 2C-D). It happened due to the loss of limonene with progressing time and very low conversion of S-(+)-carvone at these high concentrations. Therefore, 1.0 g/L of dill oil with 24 h incubation was regarded as the optimized substrate concentration and incubation period to achieve the highest abundance of *cis*-(-)-DHC in the culture broth.

Time course experiment (up to 72 h) with increasing concentration of S-(+)-carvone (0.5, 0.7, 1.0 and 1.5 g/L) further supported the optimization data. At 0.5 g/L of S-(+)-carvone [*i.e.* equivalent to ~1.1 g/L dill oil containing 47.3% S-(+)-carvone], the relative % of *cis*-(-)-DHC was highest (93.4%) in the extract after 24 h with substrate conversion of 97.9% (Fig. 2E). After 72 h, % *cis*-(-)-DHC decreased to 81.7% as observed in case of dill oil due to further reduction. At 0.7 g/L the observed rate was relatively slower. After 24 h, the abundance of *cis*-(-)-DHC reached up to 75.4% that increased to 86.7% after 48 h (highest) and then started decreasing (84.9% after 72 h) (Fig. 2F). However, at 1.0 g/L S-(+)-carvone [*i.e.* equivalent to ~2.1 g/L dill oil] the % *cis*-(-)-DHC didn't increase above 20.4% whereas in case of 1.5 g/L [*i.e.* equivalent to ~3.2 g/L dill oil] the formation of *cis*-(-)-DHC was very low (3.0%) even after 72 h (Fig. 2G-H). These results corroborated the outcome of the time course study for dill oil and indicated 0.5 g/L (24 h) as the optimized concentration of S-(+)-carvone in the current biotransformation process.

A thorough literature survey found several reports on the microbial transformation of S-(+)-carvone to *cis*-(-)-DHC (Table 2). However, lower epimeric selectivity and transformation efficiency, uncontrolled reduction and unsystematic investigation have been the major drawbacks in these studies [21–26]. For example, *Mucor circinelloides* was reported to reduce S-(+)-carvone (0.29 g/L) to *cis*-(-)-DHC as the major product (75%) in 4 h [25]. Unfortunately, other epimer (*cis*:*trans* 9:1) along with keto-reduced products like dihydrocarveol and neodihydrocarveol were also formed. The growing cells of *Diplogelasinospora grovesii*, *Gongronella butleri* and *Schizosaccharomyces octosporus* also reduced S-(+)-carvone (2.5 mM) to *cis*-(-)-DHC [22]. The abundance of *cis*-(-)-DHC reached the peak (~52–72%) after 28 h which decreased gradually due to further reduction to carveols. The formation of *trans*-(-)-DHC was also detected in case of the last two organisms. When immobilized *D. grovesii* was incubated with S-(+)-carvone (2.5 mM), > 90% conversion was achieved after 96 h with carveol as the major product (65%) and *cis*-(-)-DHC as the minor (26%) [26]. A recombinant *Escherichia coli* overexpressing *Nicotiana tabacum* enone reductase produced the dihydrocarvone with an epimeric ratio of 71:29 (*cis*:*trans*) [23]. In another study, five marine microalgae were screened for the reduction of S-(+)-carvone (1.0 g/L) and highest transformation (~65%) was obtained with *Porphyridium purpureum* after 5 days of incubation yielding only 53% *cis*-DHC [24]. The minor formation of *trans*-DHC and dihydrocarveols was also observed. In 2012 Aquino and co-workers reported the carvone reduction by *Penicillium citrinum* and *Fusarium oxysporium* [21]. *P. citrinum* produced an epimeric ratio of 84:16 (*cis*:*trans* dihydrocarvone) after 24 h. Although no *trans* isomer was detected after 24 h in case of *F. oxysporium* catalyzed reduction, the information on the progress of the reaction, % of *cis*-(-)-DHC and associated dihydrocarveols were missing.

On the other hand, *F. equiseti* in the current study was highly specific to the *cis*-epimer, efficient up to 0.5 g/L of S-(+)-carvone and further reduction could be avoided by limiting the reaction time up to 24 h. Thus, a *cis*-(-)-DHC rich product (>91% by GC-MS) was obtained making *F. equiseti* an efficient and superior biocatalyst in comparison to the previous ones. The results also revealed that either dill oil or S-(+)-carvone may be used as the substrate in this developed technique yielding very similar metabolite profile. In the case of dill oil, limonene was lost through evaporation whereas the second option saved limonene but involved an additional purification step for S-(+)-carvone. However, any future commercial producer may choose either of the substrates as the cost analysis (economy) favours.

The antifungal activity of dill oil, S-(+)-carvone and dihydrocarvones is well-known in the literature [39–43]. The ability of dill oil to disrupt the permeability of plasma membrane and cause mitochondrial dysfunction has been demonstrated in *Aspergillus flavus* [42]. It has been shown to inhibit the mycelial growth of several fungal pathogens (*Aspergillus* and *Alternaria sp.* infecting cherry tomatoes) in PDB medium [43]. A complete inhibition of the biomass production was observed at 2.0 g/L. The minimum inhibitory concentration (MIC) of S-(+)-carvone against *F. verticillioides*, a pathogen of maize was reported to be 3.84 mM (0.58 g/L) [41]. The minimum dose of S-(+)-carvone for the complete growth inhibition of various post-harvest fruit-decaying fungi was in the range 1.0–3.0 g/L in PDA [39]. Moreover, the inhibitory effect of the substrate (>0.5 g/L) and product (>0.2–0.6 g/L) was well-demonstrated while studying the biotransformation of (-)-*trans*--carveol to R-(-)-carvone by *Rhodococcus erythropolis* [44]. This information firmly supported the possibility of substrate and/or product inhibition in this process and explained the low conversion at higher concentrations. To further enhance the productivity and industrial viability of this process by overcoming the toxic effect of substrate and/or product, *in situ* substrate feeding and product removal strategy may be adopted [45,46]. For example, the productivity of (-)-*trans*--carveol conversion to R-(-)-carvone by *R. erythropolis* was enhanced by using an aqueous-organic system in a two phase partitioning bioreactor which was further improved by incorporating solid polymer beads as the partitioning medium [47]. Again, the challenges of substrate toxicity,

Table 2
Details of the previous studies on the microbial transformation of S-(+)-carvone majorly yielding *cis*-(-)-dihydrocarvone (DHC) ['-': not mentioned; Alcohol: all the keto reduced analogues of S-(+)-carvone including carveols and dihydrocarveols].

Sl. No.	Microorganism	Conc (g/L)	Time	*cis*-(-)-DHC	*trans*-(-)-DHC	*cis* : *trans* DHC	Alcohol	Residual S-(+)-carvone	Reference
1	*Mucor circinelloides*	0.29	4 h	75%	–	9: 1	–	–	[23]
2	*Diplogelasinospora grovesii* (immobilized in polyurethane foam)	0.38	96 h	26%	–	–	65%	9%	[24]
3	*Diplogelasinospora grovesii*, *Gongronella butleri*, *Schizosaccharomyces octosporus*	0.38	28 h	52–72%	no or minor	–	10–30%	0–10%	[20]
4	*Recombinant Escherichia coli overexpressing Nicotiana tabacum enone reductase*	0.20	24 h	–	–	71: 29	–	–	[21]
5	*Porphyridium purpureum*	1.00	5 d	53%	trace	–	3%	35%	[22]
6	*Fusarium oxysporium*	0.30	24 h	–	not detected	100:0	–	–	[19]
			72 h	–	–	–	major product	Full conversion	
7	*Penicillium citrinum*	0.30	24 h	–	–	84:16	–	–	[19]

low aqueous solubility, undesired isomerization and reduction were overcome by using water-immiscible ionic liquids or hydrophobic adsorbent resins for the bioreduction of R-(-)-carvone to *trans*-(+)-dihydrocarvone by a recombinant *E. coli* overexpressing ene-reductase [48]. The further scope of the current study may be directed towards tackling the challenge of substrate and/or product inhibition.

3.3. Organic solvent-free processing to cis-(-)-DHC rich hydrosol

To make an organic solvent-free downstream processing, the culture broth containing fungal biomass and metabolites (0.6 g dill oil in 600 mL broth, 1.0 g/L) was subjected to distillation. The product was isolated in the form of *cis*-(-)-DHC rich hydrosol (525 mL) through continuous collection without recirculation in a 2 L Clevenger-type apparatus (Fig. 3). The GC-MS analysis of the hydrosol revealed a qualitatively and quantitatively similar chemical profile as that of the solvent extract from the same batch of transformation. This aromatic hydrosol was considered as the final produce of the developed whole-cell biocatalyzed technique.

For further analysis, the hydrosol (325 mL) was extracted with ethyl acetate repeatedly to obtain the 'hydrosol extract' as colourless oil (155.0 mg). The absolute amount of *cis*-(-)-DHC in the hydrosol extract and hydrosol was quantified through two different techniques *i.e.* GC-MS (eugenol as the internal standard) and ^{1}H qNMR spectroscopy (1,4-dimethoxybenzene as the internal standard). The absolute abundance of *cis*-(-)-DHC in the hydrosol extract was determined as 70.00% and 70.54% respectively. Therefore, the absolute quantity of *cis*-(-)-DHC in the hydrosol was in turn, 0.33 g/L (GC-MS) and 0.34 g/L (^{1}H qNMR). The hydrosol is generally obtained as the byproduct (distillate) of hydrodistillation targeting the essential oil extraction from aromatic plant materials. It is often traded as floral water, aromatic water or herbal water. To the best of our knowledge, this is the first study demonstrating the direct production of an aromatic hydrosol through the distillation of a fermented culture.

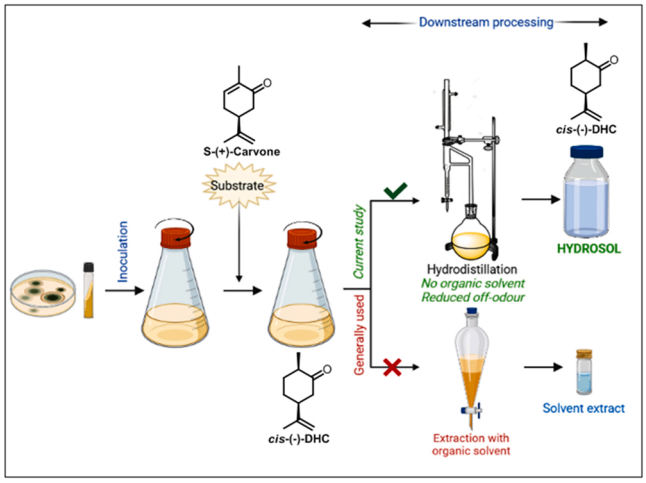

Fig. 3. Schematic representation of the organic solvent-free bioconversion of S-(+)-carvone to *cis*-(-)-dihydrocarvone (DHC) rich hydrosol developed in the current study.

3.4. Aroma profiling of cis-(-)-DHC rich hydrosol and hydrosol extract

A detailed characterization of the aroma for the produced hydrosol is the first step towards its application as the flavour or fragrance. Several sensory analyses including difference from control test, triangle test, odour threshold value determination and descriptive sensory analysis were performed to find its uniqueness in comparison to the substrate and characterize the aroma. The difference from control (DFC) test in reference to the dill oil showed moderately high difference scores for the hydrosol extract (2.06), hydrosol (2.76), *cis*-(-)-DHC (1.59) and solvent extract (2.06), indicating these products to be significantly different from the substrate in regards to their aroma (Fig. 4A). A low difference score for the blind control (0.12) validated the outcome of this experiment. In a triangle test between dill oil and hydrosol/hydrosol extract, the number of correct responses (15/16) by sixteen panelists was higher than the minimum number (9) to establish the difference (statistical significance at 5%). It confirmed the difference in aroma between the substrate and produced hydrosol/hydrosol extract and demonstrated the uniqueness of its sensory property. When the average odour threshold values (OTVs) of hydrosol extract (0.010%) and *cis*-(-)-DHC (0.029%) were determined in water, they were close to the dill oil (0.018%). The OTV of hydrosol was determined as 3.19% when diluted in water. The odour activity value (OAV) of *cis*-(-)-DHC in the hydrosol and hydrosol extract was calculated as 1.55 and 3316.90 respectively whereas for S-(+)-carvone it was 0.17 and 355.70. The OAV of *cis*-(-)-DHC was more than nine times of S-(+)-carvone; revealing its critical role in creating the overall aroma of the biotransformation product.

Descriptive sensory (odour) analysis was performed for the dill oil, hydrosol extract and solvent extract to comparatively analyze their aroma (Fig. 4B). According to the analysis, the hydrosol extract had sweet, minty, fresh, spicy and herbal note. In fact, it had a stronger sweet note in comparison to dill oil whereas minty and fresh notes were weaker in this biotransformed product. *cis*-(-)-DHC is an interesting aroma chemical and reported to possess a caraway [rich in S-(+)-carvone] like odour with herbaceous bynote. It is known to have odour similarity with S-(+)-carvone though relatively mild and sweet [13,14]. Thus, aroma profile of the produced hydrosol extract in the current study resembled the odour description of *cis*-(-)-DHC. It supported our

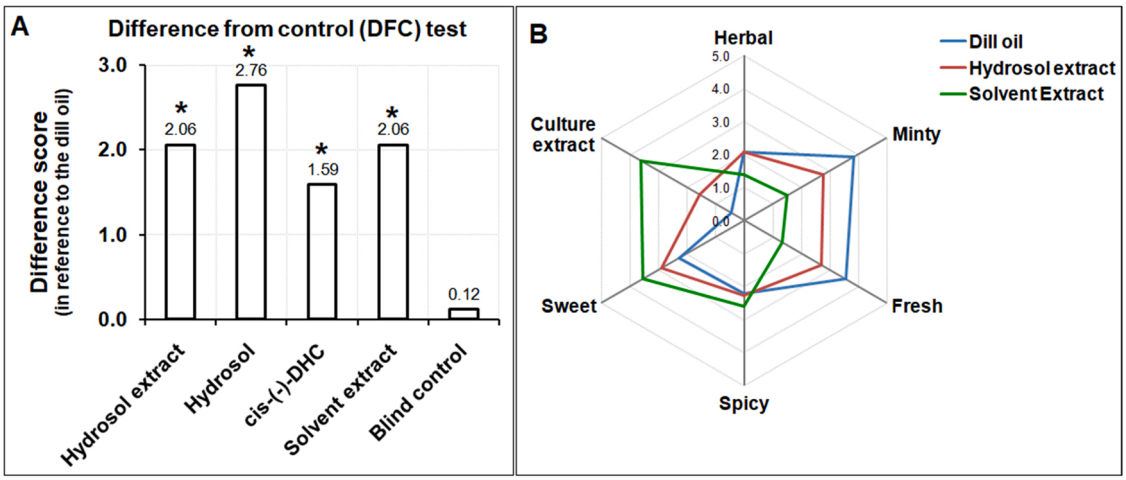

Fig. 4. Sensory analyses: (A) difference from control (DFC) test: difference scores for the hydrosol extract, hydrosol, *cis*-(-)-dihydrocarvone (DHC) and solvent extract in reference to the dill oil (*P < 0.05 vs the blind control), (B) descriptive sensory (odour) analysis scores for the dill oil, hydrosol extract and solvent extract.

inference on the key role of *cis*-(-)-DHC for the hydrosol aroma. In addition, headspace (40 °C)-GC-MS analysis of the hydrosol and hydrosol extract revealed their vapour phase composition, relating to the key odorants. It showed that the headspace composition was mainly dominated by *cis*-(-)-DHC, further supporting it as the majorly abundant key odorant responsible for the aroma of hydrosol (95.3%) or its extract (96.7%) (Fig. 1D, Table 1). Interestingly, aroma profile of the solvent extract significantly differed from the dill oil or hydrosol extract. It possessed a strong off-odour similar to the 'culture extract' which was much weaker in the hydrosol extract. A sweet note was also strongly perceived in this sample. It inferred that the distillation technique used for the extraction in this study reduced the solvent extractable off-odour (s) originated from the culture broth and fungal biomass.

4. Conclusion

In conclusion, an efficient biotransformation technique was developed for the organic solvent-free conversion of S-(+)-carvone in dill oil to *cis*-(-)-DHC rich hydrosol using *F. equiseti* as the whole-cell biocatalyst. The pH (5−6), substrate concentration (1.0 g/L) and incubation period (24 h) were optimized to achieve a nearly quantitative conversion to S-(+)-carvone metabolites, dominated by *cis*-(-)-DHC (>91%). The introduction of distillation step for the isolation of biotransformation product in the form of hydrosol eliminated the use of organic solvent and reduced the solvent extractable off-odour from the culture. The produced hydrosol containing 0.33 g/L *cis*-(-)-DHC had a unique aroma that was characterized as sweet, minty, fresh, spicy and herbal. The technique demonstrated in the current study might be useful for the production of *cis*-(-)-DHC or an aromatic hydrosol valuable in the food and flavour industries.

CRediT authorship contribution statement

Pranjit Kumar Bora: Methodology, Data curation, Writing – original draft. **Gitasree Borah:** Methodology. **Marium Begum**: Methodology. **Siddhartha Proteem Saikia:** Resources, Writing – review & editing. **Saikat Haldar:** Conceptualization, Supervision, Methodology, Writing – original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

The current work was supported by CSIR-Focused Basic Research (FBR) projects under Agriculture, Nutrition and Biotechnology theme (MLP1017) and the Director, CSIR-NEIST. We thank the National Fungal Culture Collection of India (NFCCI), Pune, India for the support related to the culture identification and deposition.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.procbio.2022.08.019.

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