International Journal of Food Microbiology 211 (2015) 44–50

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

2,4-Di-*tert*-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated *Lactococcus* sp.

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ARTICLE INFO

Article history:
Received 23 March 2015
Received in revised form 6 June 2015
Accepted 25 June 2015
Available online 2 July 2015

Keywords:
Antifungal
Antioxidant
Biopreservation
Cytotoxicity
2,4-Di-*tert*-butyl phenol
Lactic acid bacteria

ABSTRACT

The volatile organic compound 2,4-di-*tert*-butyl phenol (2,4 DTBP) was purified from the cell free supernatant of a newly isolated *Lactococcus* sp. by solvent extraction and chromatographic techniques. Molecular characterization of the compound by ESI-MS, ^{1}H NMR and FTIR analysis revealed the structure, $C_{14}H_{22}O$. Fungicidal activity was demonstrated against *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium chrysogenum* by disc diffusion assay. Among the cell lines tested for cytotoxicity of this compound (normal cell line H9c2 and cancer cell lines HeLa and MCF-7), a remarkable cytotoxicity against HeLa cells with an IC_{50} value of 10 μg/mL was shown. A biocontrol experiment with 2,4 DTBP supplemented fraction prevented growth of the abovementioned fungi on wheat grains. The study further strengthens the case for development of biopreservatives and dietary antioxidants from lactic acid bacteria for food applications.

1. Introduction

The food industry is in a continuous search for new biopreservatives where antifungal lactic acid bacteria (LAB) and their metabolites can be used effectively. LAB have received the GRAS (generally recognized as safe) and the QPS (qualified presumption of safety) status and represent the microbial group most commonly used as protective cultures (Gaggia et al., 2011). It is estimated that over 3400 tonnes of pure LAB cells is consumed every year in Europe alone (Franz et al., 2010). An array of antifungal compounds produced by LAB have been identified and characterized and include organic acids, reuterin, proteinaceous compounds, cyclic dipeptides (Oliveira et al., 2014; Schnurer and Magnusson, 2005), methylhydantoine, mevalonolactone (Niku-Paavola et al., 1999) and 2-propenyl ester (Wang et al., 2012).

Phenolic compounds as food additives are of particular interest when considering health and diet due to their antioxidant properties. The advantage of LAB metabolites having both antifungal and antioxidant properties is that they can be developed as food preservatives with dietary antioxidant properties once incorporated into food. Production of phenolic compounds by LAB and their antifungal activity is not meticulously researched and remains to be explored. LAB are capable of producing volatile phenolic compounds according to the availability of suitable substrates. It is reported that supplementation of caffeic acid, p-coumaric acid, malic acid and ferulic acid along with 5% v/v ethanol in MRS led to the production of volatile phenolic compounds, 4 vinyl phenol and 4 ethyl phenol by *Lactobacillus* through p-coumaric acid metabolic pathway (Silva et al., 2011a, 2011b). Production of 4VP and 4EP by *Lactobacillus* in wine conditions was detected by Fras et al. (2014).

Cereals comprise a major food source and contribute more than 60% to the world food production providing dietary fiber, proteins, minerals and vitamins required for human health. Beneficial effects of cereals can be exploited in different ways leading to the design of novel functional foods based on cereals or cereal ingredients that can target specific populations (Charalampopoulos et al., 2002). One of the major causes of cereal spoilage during storage is contamination by fungi which, apart from diminishing the quality and nutritive value, may produce mycotoxins that can cause severe health problems. In response to consumer demands, biopreservation technologies are being favored to improve the safety, nutritional value and the organoleptic properties of cereals (Oliveira et al., 2014). Previous investigations showed that if cereals are fermented with LAB strains, the shelf life can be further extended (Blandino et al., 2003).

A pool of different LAB strains was screened to synthesize antioxidant peptides during sourdough fermentation of cereal flours (Coda et al., 2011). However, reports on antioxidant phenolic compound production and their contribution towards antifungal and antioxidant properties of LAB were not addressed well. As a continuation of our earlier study (Varsha et al., 2014), here we report the purification and characterization

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.06.025
0168-1605/

of the phenolic compound 2,4 di-tertiary butyl phenol (2,4 DTBP) from *Lactococcus* sp. To the best of our knowledge, this is the pioneer report of a phenolic compound extracted from the culture broth of a lactic acid bacterium showing a wide spectrum of biological functions including antifungal, antioxidant and anti tumor properties.

2. Materials and methods

2.1. Microorganisms, cell lines and culture conditions

Lactococcus sp. BSN307 (NCBI accession KM261818), previously denoted as BSN in Varsha et al. (2014) was cultivated in MRS (de Man Rogosa Sharpe, Himedia, India) medium at 30 °C for 24 h and preserved at −20 °C in MRS broth with 20% (v/v) glycerol. The fungal test strains, *Aspergillus niger* (KACC 42589), *Penicillium chrysogenum* (NII 08137), *Fusarium oxysporum* (KACC 42109), *Fusarium moniliforme* (KACC 08141), *Fusarium graminearum* (MTCC 1893), *Fusarium chlamydosporum* (MTCC 2399) were maintained on potato dextrose agar (Himedia, India) at 30 °C. Mammalian cell lines, H9c2 (myoblast cell line), HeLa (cervical adenocarcinoma cell line) and MCF-7 (breast carcinoma cell line) were obtained from ATCC (American Type Culture Collection).

2.2. Chemicals and solvents

All the organic solvents and H_2SO_4 used in this study were obtained from Merck (India), DPPH (1,1-diphenyl-2-picryl-hydrazyl), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), Dulbecco's Modified Eagle's Medium (DMEM) and trichloroacetic acid, streptomycin, penicillin and amphotericin B were purchased from Sigma (India). Analytical grade sodium sulfate, ammonium molybdate, potassium ferricyanide, ferric chloride, fetal bovine serum (FBS) and ascorbic acid were purchased from Himedia (India).

2.3. Purification and characterization of the bioactive

For purification, 10 L of MRS medium was inoculated (5% (v/v)) with 24 h old culture and incubated at 30 °C for 48 h under static conditions. After incubation, the supernatant was collected by centrifugation at 8590 ×*g* for 15 min and extracted with double volume of chloroform. The solvent was evaporated under reduced pressure in a Rotavapor (Buchi, Switzerland) and the concentrated sample was further dissolved in acetonitrile (B1 fraction). The B1 fraction was purified by high pressure liquid chromatography (Shimadzu UFLC, Japan) using Prep-C18 column (Phenomenex, US) and acetonitrile: water (90:10) as mobile phase with a flow rate of 4 mL/min. Fractions with antifungal activity were pooled and purified further by analytical HPLC (C18 column, Phenomenex, US) using acetonitrile as mobile phase with a flow rate of 1 mL/min. Antifungal activity of the purified product (400 μg/disc) was confirmed against *F. oxysporum* (KACC 42109) by disc diffusion assay as described in Section 2.4.

2.3.1. Structure analysis

Structure of the antifungal compound was determined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy using Bruker Avance II 500 spectrometer (US). Liquid chromatography–mass spectrometry (Thermo Orbitrap LC/MS, US) employing electrospray (ESI) ionization method was used to determine molecular mass of the compound. The sample was prepared in acetonitrile with 0.1 N formic acid and acetonitrile was used as mobile phase. Fourier transform infrared spectroscopy (FTIR) was performed with PerkinElmer model Spectrum 100 (US) to obtain IR spectrum of the compound.

2.4. Antifungal activity assay of the pure compound

As well as *F. oxysporum* (KACC 42109), the wide spectrum antifungal activity of the purified compound (400 μg) dissolved in 50% acetonitrile was checked against *A. niger* (KACC 42589), and *P. chrysogenum* (NII 08137) by disc diffusion method on PDA plates which were already swabbed with individual fungal suspension (1×10^4 spores/mL). The control was 50% acetonitrile and the plates were incubated at 30 °C for four days to check the inhibition zones.

2.5. Total antioxidant and ferric Ion (Fe^{3+}) reducing activities

Active B1 fraction (1 mg/mL) containing 2,4 DTBP was checked for its total antioxidant and Ferric Ion (Fe^{3+}) reducing activities by the methods previously reported by Prieto et al. (1999) and Oyaizu (1986) respectively and the activities were expressed as ascorbic acid equivalents.

2.6. Assay of free radical scavenging

Free radical scavenging activity of the B1 fraction as well as 2,4 DTBP (≥90% purity) purified from BSN307 culture medium were determined by the method of Shimada et al. (1992) which is based on the principle of scavenging the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical. DPPH was prepared in 95% ethanol to a final concentration of 0.2 mg/5 mL. Samples were prepared in methanol with the concentration of B1 fraction as 5 mg/mL and 2,4 DTBP as 2 mg/mL and the radical-scavenging activities of samples were expressed as percentage scavenging of DPPH. Each mixture was kept in the dark for 30 min and the absorbance was measured at 517 nm against a blank. The scavenging ability was defined as:

$$\text{Scavenging activity } (\%) = (A_{\text{Control}} - A_{\text{Sample}})/A_{\text{Control}} \times 100.$$

2.7. Cytotoxicity to mammalian cell lines

Cytotoxicity against mammalian cells was determined by MTT assay as described previously by Mosmann (1983), where the conversion of tetrazolium salt MTT to blue colored formazan by the mitochondrial enzyme succinate dehydrogenase which is active in living cells was measured to quantify cell survival and proliferation. In brief, mammalian cell lines, H9c2, HeLa and MCF-7 were maintained in DMEM supplemented with 10% FBS, 10 mg/L streptomycin, 100 U/L penicillin and 25 μg/mL amphotericin B. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 (SANYO CO_2 Incubator, Japan) until a confluent monolayer was obtained. Cells were seeded at a concentration of 1×10^4 cells into each well of a 96-well tissue culture plate followed by incubation at the same conditions for 24 h. The medium was removed and the cells were treated with varying concentrations of purified 2,4 DTBP prepared in DMEM without FBS and incubated in the CO_2 incubator for 24 h. After incubation, the medium was removed from each well with a pipette and 200 μL of MTT (0.5 mg/mL) dissolved in DMEM was added followed by incubation in the dark for 3 h. The reaction was stopped by addition of 100 μL DMSO into the wells. Plates were further incubated for 30 min in a shaker and the absorbance was read at 570 nm. The experiments were performed in triplicate. Cytotoxicity (%) and IC_{50} (effective concentration of drug resulting in 50% of maximal toxicity) were calculated as follows:

$$\text{Cytotoxicity}(\%) = [1 - (A_{\text{Test}}/A_{\text{Control}})] \times 100.$$

2.8. Biocontrol of fungi on wheat grains

The wheat grains were surface disinfected with 4% sodium hypochlorite and thoroughly washed with sterilized distilled water followed by air drying. The active fraction (B1) was extracted from 1 L of BSN307 culture filtrate using double volume of chloroform, concentrated in a rotary evaporator and dissolved in 20% acetonitrile.

Grains were treated with active fraction (25 mg/mL) for 15 min and air dried along with those treated in 20% acetonitrile as control. The dried grains were distributed into sterile glass vials and inoculated with 2–3 day old mycelial plugs (~5 mm) of *A. niger*, *F. moniliforme*, *F. graminearum*, *F. chlamydosporum* and *F. oxysporum* and incubated at room temperature. Fungal growth on the grains was monitored every day.

2.9. Statistical analysis

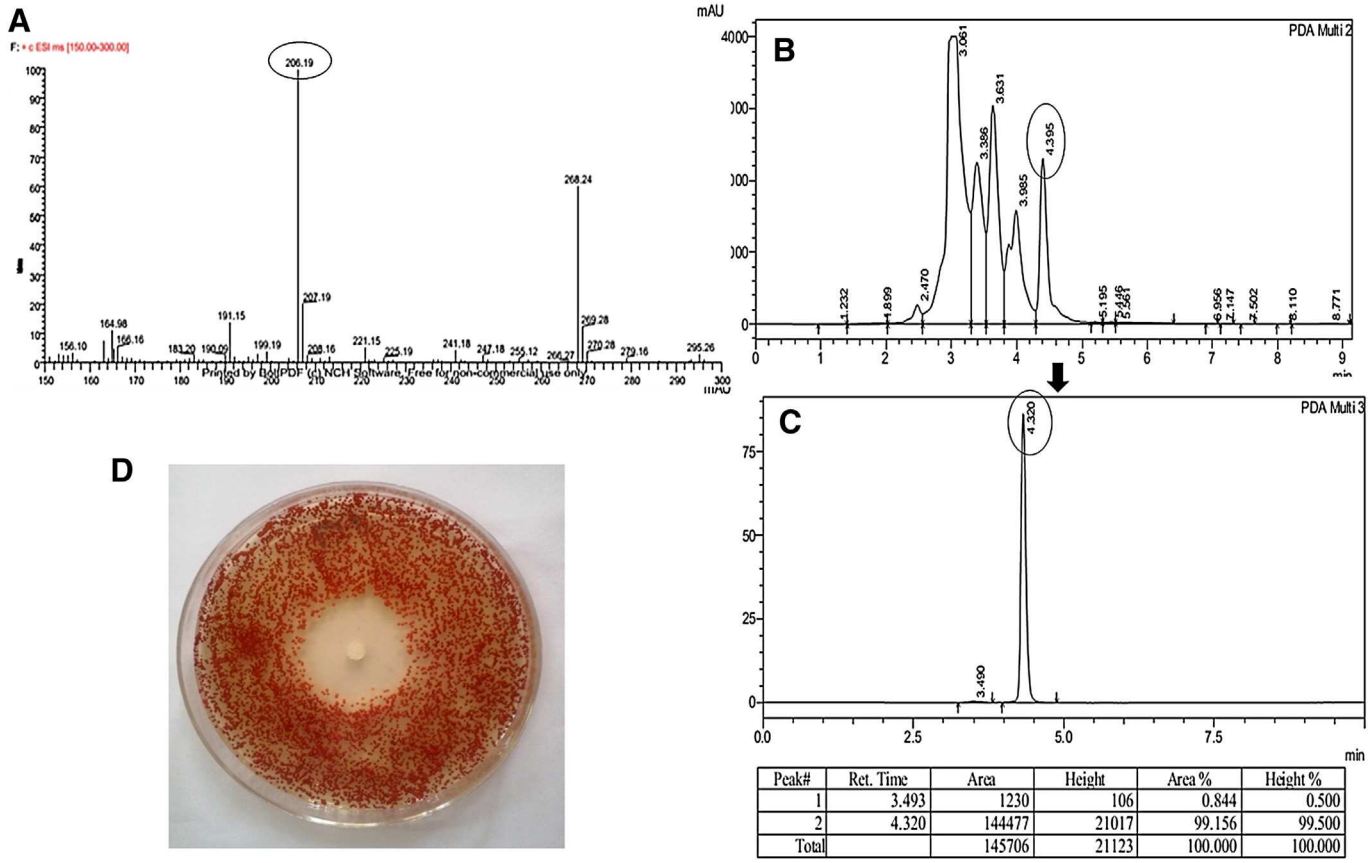
All the experiments were performed in triplicate and results were expressed as mean values ± standard deviation. One-way ANOVA and Dunnet's test were performed to test the differences between test and control groups ($p < 0.05$). Statistical analyses were performed using the Minitab statistical package v. 17 (Minitab Inc., USA).

3. Results and discussion

3.1. Purification and characterization

Solvent extraction was followed in a sequential order, from hexane–chloroform–ethyl acetate. No antifungal activity was observed in the hexane fraction and the antifungal compounds identified in ethyl acetate have been previously reported (Varsha et al., 2014). The remaining solvent fraction with activity was the chloroform fraction and this was further purified to identify the antifungal compounds. HPLC analysis of prep-C18 purified sample revealed a major component of 90% and few minor impurities contributing the remaining 10%. The sample was then subjected to LC–MS analysis which indicated a molecular weight of 206 at positive mode corresponding to the major peak in the sample (Fig. 1A). The sample was then further purified to 99.5% by analytical HPLC using C18 column. Fig. 1B shows the HPLC chromatogram of crude B1 fraction and the purified compound (Fig. 1C) at 270 nm. NMR and IR were performed. IR spectra of a purified sample showed a peak at 3533 cm^{-1} indicating stretching of O–H phenolic group. Peaks at 2871–2959 cm^{-1} indicated C–C stretching of alkyl group. C–O stretching of phenols was seen at 1252 cm^{-1}. Presence of aromatic C═C stretching was identified by peaks at 1506–1600 cm^{-1}. Thus the presence of this functional group provided suggestive evidence that the compound is phenolic in nature. NMR data of the purified sample revealed the presence of aromatic hydrogen corresponding to 7 to 7.3 ppm, a doublet at 7.07 ppm indicate aromatic metahydrogen. Singlets of 9 hydrogen atoms at 1.309 and 1.431 suggested presence of di substituted tertiary butyl group. A downfield singlet at 4.662 indicates the presence of phenolic hydrogen (Fig. 2A). Thus, after careful interpretation and co-relation of IR, NMR and LC–MS data, the compound was structurally elucidated as $C_{14}H_{22}O$ (Fig. 2B) known as 2,4 di-*tert* butyl phenol (2,4 DTBP), a volatile organic compound (VOC) and discovered to play a major role in the antifungal activity of chloroform fraction along with other unidentified antifungal compounds. The final yield of 2,4 DTBP (≥90% purity) was 2 mg/L. Using purified 2,4 DTBP a clearing zone of 3 cm against *F. oxysporum* (Fig. 1D) was obtained.

2,4 DTBP is used as an intermediate in for the preparation of UV stabilizers and antioxidants as well as in the manufacture of pharmaceuticals and fragrances (Choi et al., 2013). The possible precursor and the pathway that leads to the production of this compound is not yet elucidated clearly although the gene clusters and enzymes required for shikimate and mevalonate pathway have been identified in *Lactobacillus* (Bolotin et al., 2001; Smeds et al., 2001).



Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.493	1230	106	0.844	0.500
2	4.320	144477	21017	99.156	99.500
Total		145706	21123	100.000	100.000

Fig. 1. Purification and antifungal activity of 2,4 di-*tert*-butyl phenol. MS chromatogram of 2,4 DTBP (standard) (A), HPLC chromatogram of B1 fraction (B) purified 2,4 DTBP (C) and antifungal activity of 2,4 DTBP against *F. oxysporum* (D).

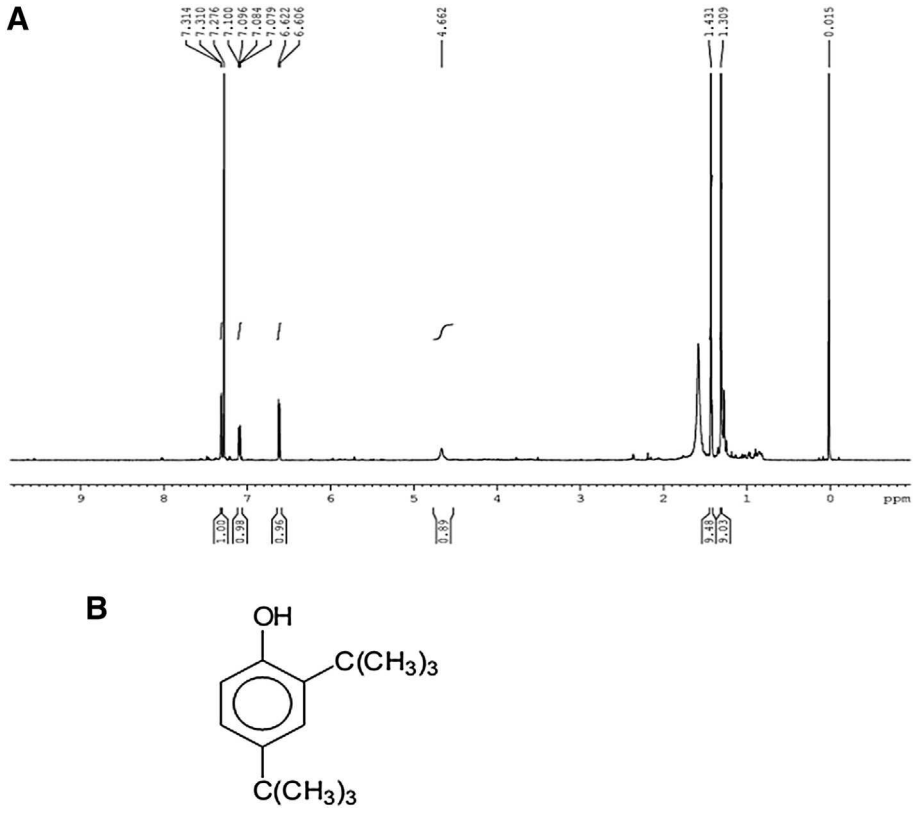


Fig. 2. Structure elucidation of 2,4 di-*tert*-butyl phenol. ^{1}H NMR spectrum (A) structure of 2,4 DTBP (B).

3.2. Antifungal and antioxidant activity assay of 2,4 DTBP

2,4 DTBP purified from the culture medium of BSN307 exhibited fungicidal activity against all the fungal strains tested. Three different genera of fungi that often cause contamination in various food products were selected to study the antifungal activity of 2,4 DTBP and all were found to be sensitive. A 400 μg/disc produced a clearing zone of 0.8 cm against *A. niger* and 1.5 cm against *P. chrysogenum*. Among the fungi tested *F. oxysporum* was most sensitive to 2,4 DTBP. The minimum inhibitory concentration against *F. oxysporum* was 62 μg/disc and produced a clearing zone of 0.6 cm.

Our result supports and confirms previous research on the antifungal activity of VOC produced by bacteria (Yuan et al., 2012; Weisskopf, 2014). 2,4 DTBP has been reported to be present in fruits as well as seeds and imparts antioxidant properties (Choi et al., 2013). This volatile phenolic compound is reported to have in vitro antimalarial activity where the plasmodial growth was arrested with an amount of 100 mM compound (Kusch et al., 2011). One recent report indicates the presence of 2,4 DTBP in the culture filtrate (CF) of an antifungal *Lactobacillus* strain where the compound was detected by GC–MS analysis (Sangmanee and Hongpattarakere, 2014). Dharni et al. (2014) reported prevention of spore germination of *F. oxysporum* with 100 μg/mL of 2,4 DTBP. The proposed mechanism of antifungal activity as described by this group was the inhibition of spore germination by preventing the emergence of normal germ tube leading to abnormal swelling and branching of hyphae. It has been demonstrated that 2,4 DTBP inhibits the assembly of spindle microtubules and disturbs the chromosomal alignment at the metaphase plate and microtubule–kinetochore interactions, causing chromatid loss, which may reduce the mycelia growth and the germination of spores. Our study confirms the earlier reports as well as describes the efficiency of this compound in prevention of *A. niger* and *P. chrysogenum* growth along with growth inhibition of *F. oxysporum* and points towards the possibility of production of other VOC with various biological activities from LAB.

Evaluation of total antioxidant capacity (TAC) by the phosphomolybdenum method showed that 40 μg of B1 fraction had 80% antioxidant activity where the same amount of ascorbic acid had 83% activity. Ferric ion reducing power assay revealed that 1 mg of this fraction held 33% of reducing power and the same amount of ascorbic acid showed 50% activity.

3.3. Assay of free radical scavenging

The percentage of free radical scavenging activity of the B1 fraction and 2,4 DTBP is shown in Fig. 3. Fourty micrograms of purified 2,4 DTBP had 77.5% free radical scavenging ability. 2,4 DTBP was reported to be associated with the antioxidant effect of the methanolic extract

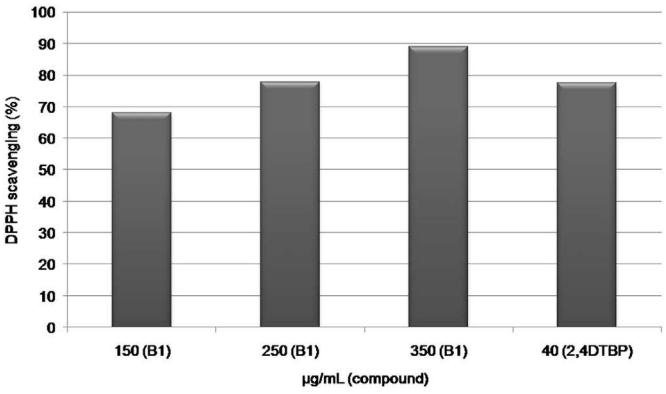


Fig. 3. DPPH scavenging activity of different concentrations of B1 fraction and pure 2,4 di-*tert*-butyl phenol ($p < 0.05$).

from dried *Scolopendra subspinipes mutilans* (Scolopendridae), a centipede that has been utilized as a traditional Chinese and Korean medicine for a variety of diseases, such as spasm, seizures, poisonous nodules, childhood convulsions, diphtheria and tetanus. The antioxidant effects displayed by this compound include prevention of LDL oxidation and DPPH scavenging ability (Yoon et al., 2006). In a study by Choi et al. (2011), the ethanol extract of pomegranate had antioxidant and neuronal protective effects and diminished hydrogen peroxide induced oxidative stress in PC-12 cells. The protective effect against learning and memory impairment induced by $A\beta_{1-42}$ is due to the antioxidant effect of 2,4 DTBP where ABTS radical assay was followed to confirm the antioxidant activity of 2,4 DTBP. The disease prevention roles of fruits, vegetables and red wine have been attributed, in part, to the antioxidant properties of their constituent polyphenols (vitamins E and C, and the carotenoids) and the antioxidant property of plant materials was found to increase with an increase in the amount of total phenols (Rice-Evans et al., 1997; Velioglu et al., 1998). Apart from the excellent antifungal activity, the antioxidant activity assays revealed the potential of this fraction to act as reducing agent and thus inhibit the oxidation of molecules as well produce free radicals. Based on this study and also the previous studies 2,4 DTBP has been shown to be a natural antioxidant with very good protective effect against oxidative damage.

3.4. Assay of cytotoxicity to cancer cell lines

With the crude supernatant no cytotoxic activity was observed. However, 2,4 DTBP purified from the culture medium displayed remarkable cytotoxic activity against HeLa cell lines with an IC_{50} value of 10 μg/mL. IC_{50} against MCF-7 was achieved with 16 μg/mL. Against the normal cell line H9c2, a higher amount of 19 μg/mL was required to bring about IC_{50}. The values are represented in Fig. 4. The cytotoxic property of 2,4 DTBP has been previously reported by different research groups. Malek et al. (2009) purified 2,4 DTBP from the leaves of *Pereskia bleo* (Kunth) where it represents a major component and found it to have a cytotoxic effect on the human cancer cell lines KB, MCF-7, CasKi, HCT 116, A549 and in normal human cell line MRC-5. However, HeLa and H9c2 cell lines were not included there and were covered in this study. In the abovementioned study, IC_{50} against MCF-7 was obtained with 5.75 μg/mL and in MRC-5 with 20 μg/mL. At the same time 29 μg/mL was required in HCT 116 cell line and below 6 μg/mL was enough to bring about IC_{50} in other cell lines used. Leaves of Kunth are used traditionally in Malaysia for the treatment of cancer, diabetes, high blood pressure and diseases associated with rheumatism and inflammation as well as remedy for the relief of gastric pain, ulcers and for revitalizing the body. Presence of this compound in plants is not yet explained biogenetically but the same authors (Malek et al., 2009) reported the presence of 2,4 DTBP in the termite-associated fungus *Termitomyces heimi* and the edible fungus *Hericium erinaceus*.

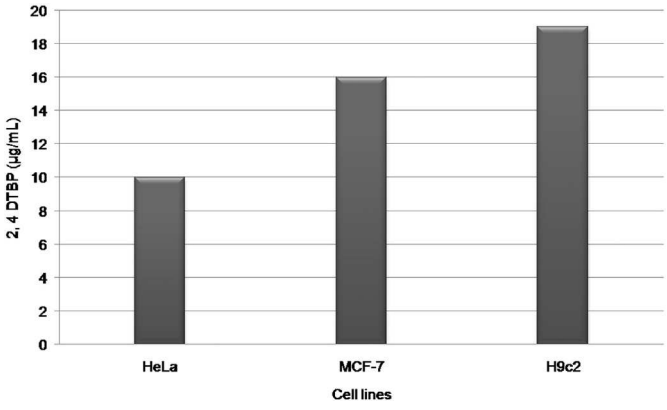


Fig. 4. IC_{50} of 2,4 di-*tert*-butyl phenol in μg/mL against different cell lines ($p < 0.05$).

In a study by Choi et al. (2013), sweet potato extract is reported to have protective effect against hydrogen peroxide-induced oxidative stress and cytotoxicity on the pheochromocytoma cell line (PC12). The active component in this extract was purified and identified as 2,4 DTBP and administered to check the effects on amyloid-beta peptide ($A\beta_{1-42}$) induced learning and memory impairment in mice and shown to have a protective effect against $A\beta_{1-42}$ by decreasing neuronal cell damage and increased spontaneous alteration behavior in mice when provided a diet supplemented with 2,4 DTBP. A previous study (Chen et al., 2007), reported the effect of six day old extracts of fermented milk product kefir reduced the growth of human mammary cancer cells (MCF-7) in a dose-dependent manner, showing 29% inhibition of proliferation at a concentration of 0.63% without any antiproliferative effect against normal human mammary epithelial cells (HMECs). Even though it has certain degree of cytotoxicity in normal cell lines, studies show that 2,4 DTBP is a common ingredient in many medicinal preparations and can be helpful during disease conditions.

The presence of 2,4 DTBP in the cell free supernatant (CFS) of LAB may be one of the reasons behind their many health benefits including anticancer properties. LAB represent a major component of the human microbiota and are common starter cultures in food fermentations. Since LAB are normal part of our daily diet, their intake may carry therapeutic effects as they are source of natural antioxidants and may be particularly useful when long term treatment is required.

3.5. Biocontrol of fungi on wheat grains

In all vials of wheat grains treated with B1 fraction (approximately 2 mg/25 mL of 2,4 DTBP) (Fig. 5) fungal mycelial growth was completely prevented. The inoculated mycelia plug failed to start growing and dried off. In the control vials, fungal mycelia started growing on the third day of inoculation and by seven days infested the grain leading to spoilage. *F. chlamydosporum* and *F. oxysporum* formed a mat on the grain surface (Fig. 5). As microbial contaminants are found mostly on the grain surface (Oliveira et al., 2014), the compounds produced by LAB may control their attack in a natural way. Due to the GRAS status, it is possible to obtain regulatory approval for the compounds produced by LAB because of their origin and incorporation into food products. Biocontrol activity of 2,4 DTBP produced by *Flavobacterium johsoniae* strain GSE09 against *Phytophthora capsici* in pepper was reported by Sang and Kim (2012). Growth of the pathogen was reduced to half with 100 μg/mL of this bioactive compound and the study demonstrated that radicle formation in pepper seeds was not affected by 2,4 DTBP treatment which protected against the infection by *P. capsici*. These authors also showed that pepper seeds could germinate after treatment with 2,4 DTBP.

Recently antimicrobial peptides from LAB were used to prevent fungal attack on wheat grains along with maintaining the health of grains under storage (Gupta and Srivastava, 2014). Thus, research by various groups has shown that 2,4 DTBP is not an unusual compound and occurs naturally in fruits, seeds and also is produced by microorganisms. LAB producing this compound can be explored and developed as starter cultures for fermented food and may also be used to prevent fungal contamination of food.

4. Conclusion

Antifungal and antioxidant property of 2,4 DTBP, obtained from a *Lactococcus* sp. demonstrate the potential of this compound for development as a food additive which may improve the food safety as well as having health promoting characteristics. This would be of particular interest in grain or cereal based food products where fungal contamination is a major cause of food spoilage. Cytotoxicity against cancer cell lines revealed the diverse biological potential possessed by this bioactive and provides evidence on the therapeutic potential of lactic acid bacteria.

Fig. 5. Biocontrol of fungi on wheat grains (14th day). Antifungal activity of B1 fraction containing 2,4 di-*tert*-butyl phenol against *A. niger* (A), *F. chlamydosporum* (B), *F. moniliforme* (C), along with untreated control (C) on right side.

Acknowledgments

Research fellowship from Council of Scientific and Industrial Research (CSIR), New Delhi, India to KKV was greatly acknowledged. Help from Mr. Rajaram Patil, Thermo Fischer scientific, Mumbai for LC–MS analysis and Mr. Sinu C R for FTIR analysis are greatly appreciated. Authors also thank CSIR grant under XIIth Five Year Plan Network Project, FUNHEALTH (CSC 0133).

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