

Lindane degradation by *Candida* VITJzN04, a newly isolated yeast strain from contaminated soil: kinetic study, enzyme analysis and biodegradation pathway

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Abstract A new yeast strain was isolated from sugarcane cultivation field which was able to utilize lindane as sole carbon source for growth in mineral medium. The yeast was identified and named as *Candida* sp. VITJzN04 based on a polyphasic approach using morphological, biochemical and 18S rDNA, D1/D2 and ITS sequence analysis. The isolated yeast strain efficiently degraded 600 mg L⁻¹ of lindane within 6 days in mineral medium under the optimal conditions (pH 7; temperature 30 °C and inoculum dosage 0.06 g L⁻¹) with the least half-life of 1.17 days and degradation constant of 0.588 per day. Lindane degradation was tested with various kinetic models and results revealed that the reaction could be described best by first-order and pseudo first-order models. In addition, involvement of the enzymes viz. dechlorinase, dehalogenase, dichlorohydroquinone reductive dechlorinase, lignin peroxidase and manganese peroxidase was noted during lindane degradation. Addition of H₂O₂ in the mineral medium showed 32 % enhancement of lindane degradation within 3 days. Based on the metabolites identified by GC–MS and FTIR analysis, sequential process of lindane degradation by *Candida* VITJzN04 was proposed. To the best of our knowledge, this is the first report of isolation and characterization of lindane-degrading *Candida* sp. and elucidation of enzyme systems during the degradation process.

Keywords *Candida* · Enzymes · Kinetics · Lindane · Pathway

Introduction

Lindane (γ -HCH), an organochlorine compound is one of the most important pesticides used in agriculture during the last half century. It is synthesized by photochemical chlorination of benzene under UV light, along with the formation of four major stereo isomers (α , β , γ and δ HCH) (Phillips et al. 2005). Out of the four isomers, lindane is the only one with insecticidal property (Manickam et al. 2007). Lindane is listed as a priority pollutant by USEPA as it is seen as a possible carcinogen. The half-life period for lindane in soil and water was reported as 708 and 2,292 days respectively (Beyer and Matthies 2001). Although nowadays the use of lindane is restricted or altogether banned in most countries, residues of lindane are found all over the world in soil, water, air, plants, agricultural products, animals, food and humans (Pinerio et al. 2007; Kidd et al. 2008; Herrero-Mercado et al. 2010; Fuentes et al. 2011; Alvarez et al. 2012).

Biodegradation is a more efficient and environmentally friendly method for detoxification of hexachlorocyclohexane (HCH) residues compared to physical and chemical methods (Zhang et al. 2010). A number of genera of bacteria (Chaudhary et al. 2006; Manickam et al. 2007; Zhang et al. 2010; De Paolis et al. 2013), filamentous fungi (Dritsa et al. 2009; Sagar and Singh 2011; Guillén-Jiménez et al. 2012), actinomycetes (Fuentes et al. 2011; Alvarez et al. 2012) and microalgae (Zhang et al. 2012) have been reported with the ability to degrade lindane. But the reports are scanty on the potentiality of yeast as lindane degrader. In our previous work, we reported a yeast strain, identified as *Rhodotorula* sp. VITJzN03 with lindane degradation potentiality in liquid

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Table 1 Selected physico-chemical properties of the soil used for isolation of yeast, VITJzN04

Soil properties	
pH	7.68
Electrical conductivity ms cm^{-1}	62
Organic matter (%)	3.26
Nitrate nitrogen (ppm)	63
Available phosphorus (ppm)	108
Potassium exchangeable (ppm)	45
Sand (%)	41
Silt (%)	33
Clay (%)	20
Texture (as per USDA)	Loamy sandy soil

media (Salam et al. 2013). Removal of lindane depends on specific environmental conditions in the locality and on the biodegradation potential of the used microbial population. The knowledge of degradation kinetics enhances the understanding of lindane degradation and contributes towards the development of an appropriate in situ bioremediation strategy. It is further discovered that most studies lack information regarding the enzymatic mechanism of lindane degradation. A detailed study on enzymatic mechanisms and degradation pathway is thus an essential requirement for the successful implementation of microbes.

Therefore, the objectives of the present study were: (1) to isolate and identify novel yeast strain having potentiality to degrade high concentration of lindane (2) to study the kinetics of lindane degradation (3) to elucidate the involvement of key enzymes in the degradation and (4) to propose the possible metabolic pathway of lindane degradation.

Materials and methods

Chemicals

Lindane (γ -HCH, >99.9 % purity) and all enzyme substrates were purchased from Sigma-Aldrich (USA) while methanol, acetone and ethyl acetate were procured from SRL, India Ltd. A stock solution of lindane was prepared using 10^4 mg lindane dissolved in 100 mL methanol. The media components used for culture media preparation were purchased from Hi-media India Ltd. All the other chemicals used throughout the study were of high quality, obtained from standard manufacturers.

Soil Sampling

For isolation of lindane degrading yeast, rhizospheric soil was collected from sugarcane fields at Villianur ($11^\circ 55' \text{N}$,

$79^\circ 45' \text{E}$), Pondicherry, India, which had a long history of lindane application for more than 15 years. Soil cores (0–20 cm) taken from selected spots were collected in sterile plastic bags. The physico-chemical property of the soil was analyzed from the National Agro Foundation, Chennai and listed in Table 1.

Enrichment culture for isolation and screening of lindane degrading yeast strain

Yeast isolation was carried out from the rhizospheric soil samples by standard enrichment technique. The experiments were conducted in 250 mL Erlenmeyer flasks with 100 mL Mineral medium (MM; contained per litre-ammonium sulfate; 5 g, potassium dihydrogen phosphate; 1 g, dipotassium hydrogen phosphate; 2 g, magnesium sulfate; 0.5 g, sodium chloride; 0.1 g, manganese chloride; 0.01 g, ferrous sulfate; 0.01 g, sodium molybdate; 0.01 g, at $\text{pH } 7.2 \pm 0.5$) and 10 mg L^{-1} lindane. 5 g of the soil sample was added and incubated for 5 days at $28 \pm 2^\circ \text{C}$ in rotary shaker. 10 mL of the enrichment culture was transferred every 5 days to fresh sterile medium, incubated under above conditions and the concentration of lindane in the enrichment culture was increased from 10 to 100 mg L^{-1} in a step wise manner. After 2 weeks evaluation period, the spread plate and sub-culture method were used to obtain pure isolates. The isolate thus obtained was named VITJzN04 and maintained on yeast extract peptone dextrose (YEPD) agar slants with lindane (100 mg L^{-1}) at 4°C .

Identification of the yeast strain

The lindane degrading yeast was morphologically and physiologically characterized, classified and further identified in a polyphasic approach. Primary morphological characterization was done using bright field microscopy and atomic force microscopy. The isolate which resembled like yeast were further subjected to biochemical characterization using Vitek-2, compact yeast card reader with software version V2C 1.01 (CFRD, Kerala, India).

Taxonomic characterization of the yeast strain

The yeast strain was characterized and identified based on microscopic, cultural characteristics and rDNA (18S, ITS regions and D1/D2 domains) gene analysis. The genomic DNA of the isolated yeasts was amplified by using the primers, UL18F:5'-TGTACACACCGCCCGTC-3' and UL28R:5'-ATCGCCAGTTCTGCTTAC-3' (universal primers; both designed by Acme progen biotech India pvt ltd). The purified PCR products were characterized by partial and complete sequence analysis of the genes. The resulting consensus rDNA sequences were then compared to the

non-redundant NCBI database using BLAST similarity searches (Altschul et al. 1990). A collection of closely related sequences from this database was used to perform multiple alignment analysis using the CLUSTAL X program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al. 2011). The partial and complete rDNA sequences of the lindane degrading yeasts were submitted to the GenBank database under the accession number JX454449.

Batch cultures of yeast strain in mineral medium supplemented with lindane

Yeast inoculums were prepared in YEPD broth. For inoculum preparation, 48 h cultures were pelleted out, washed with phosphate buffer and resuspended into MM supplemented with lindane (100 mg L⁻¹) as sole carbon and energy source. The cultures were incubated for 10 days. After incubation, the cell suspension was centrifuged at 10,000×g for 10 min. The pellet was washed with methanol to remove residues of lindane. Then it was transferred into pre weighed Petri dishes and dried to constant weight at 105 °C for 45 min and the dry weight of biomass was calculated.

To study the effect of environmental factors on the growth and lindane degradation by the yeast, the flasks with 25 mL MM containing 100 mg L⁻¹ lindane was inoculated with 1 mL of YEPD broth culture of VITJzN04 (OD₆₀₀ = 0.1) and incubated at 28 ± 2 °C on a rotary shaker for 10 days. The effect of the pH of the medium (3–8), incubation temperature (20–40 °C), inoculum dosage (0.02–0.1 g L⁻¹ dry weight) and initial lindane concentration (200–1,000 mg L⁻¹) were studied. The tests were conducted in triplicates. Abiotic and biotic controls were maintained for each experiment.

Kinetics studies on lindane biodegradation by VITJzN04

Biodegradation kinetic studies for VITJzN04 were performed in 100 mL flasks in triplicates. Each flask contained 25 mL MM spiked with lindane (600 mg L⁻¹) and optimized dosage yeast inoculum. The broth cultures were incubated in an orbital shaker (120 rpm) at 30 °C for 8 days. The flasks were removed at required intervals for analyses of inorganic chloride, residual substrate lindane, and intermediary metabolites. The residual lindane in the culture medium was calculated using the formula:

$$\text{Residual lindane (\%)} = \left(\frac{C_t}{C_0} \right) \times 100 \quad (1)$$

where, C₀ is the initial concentration of lindane in the medium and C_t is the concentration at time t.

The zero order (Petrucci et al. 2007), first order (Dykaar and Kitanidis 1996), second order (Capellos and Bielski

1972) and pseudo first order (Capellos and Bielski 1972) kinetic models were used to define the lindane degradation in mineral medium.

Effect of H₂O₂ on lindane degradation

Since H₂O₂ has been commonly used to facilitate the degradation of chemical pollutants, especially some of the persistent organic pollutants, the effect of H₂O₂ in the enhancement of lindane degradation by VITJzN04 was studied. In this experiment, the H₂O₂ levels were set as 0, 1, 5, 10, 12 m mol L⁻¹ and added to MM with the yeasts, incubated under the optimized condition on a rotary shaker. Cultures were withdrawn on the 3rd day for estimation of lindane degradation. Controls were run simultaneously with lindane and H₂O₂ to assess the effect of lindane removal by H₂O₂ alone.

Enzyme assays

The activities of lindane degrading enzymes were checked in the yeast culture at different time intervals. The yeast cells were grown in the MM with 100 mg L⁻¹ lindane as carbon and energy source. For cell-free extracts, cells were harvested and washed twice in 100 mM Tris–HCl (pH-7.4) and resuspended in the same buffer. The re-suspended cells were incubated in ice, followed by sonication in three pulses of each 10 s. This cell free extract was used for all enzyme assays. The enzyme activity in culture supernatant was also estimated simultaneously. The crude extracts from yeast cells grown in MM without lindane was used as controls.

The enzymes viz. lindane dechlorinase and lindane dehalogenase were assayed following the method described by Zhang et al. (2009) with minor modifications using lindane and 1-chlorobutane (1-CB) as substrates respectively. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of chloride ion per min under the experimental conditions. The release of chloride ion was monitored according to the procedure of Bergmann and Sanik (1957). 2,5-dichlorohydroquinone (2,5-DCHQ) reductive dechlorinase activity was assayed using 2,5-DCHQ as substrate (Miyachi et al. 1998). Laccase (Lac) activity was determined by the oxidation of ABTS method (More et al. 2011). Lignin peroxidase (Lip) and Manganese peroxidase (MnP) activities were determined as described by Hussaini et al. (2011), using veratryl alcohol and MnSO₄ as substrates.

Analytical methods

Colorimetric assay for lindane dechlorination

Mineralization of lindane by yeast isolate was confirmed by estimating the release of free chloride ion by mercuric thiocyanate method (Bergmann and Sanik 1957). 2 mL of

cell free supernatant from each of the triplicates were removed after regular intervals for the estimation of free. The color developed was measured at 460 nm by a spectrophotometer (UV-1800-Shimadzu). The amount of chloride was computed from calibration curve using NaCl as standard.

Extraction and characterization of degraded products by GC-MS and FTIR

Residual lindane and the degraded products in the culture broths were determined by GC-MS and FTIR analysis. Flasks were withdrawn at regular intervals for analysis of degraded products. The residual lindane as well as the degradation products were extracted and analysed by GC-MS as described by Salam et al. (2013). The spectra were compared with respective spectra of authentic compounds and also with the mass profile of the same compound available in the National Institute of Standard Technology (NIST) library, USA.

The FTIR spectra of lindane and degraded products were used to determine the vibrational frequency changes in functional groups. The extracted degraded products dissolved in ethyl acetate, were mixed with KBr and made in the form of pellets (13 mm in diameter and 1 mm thickness). IR spectroscopy was investigated with an IR affinity-1 FT-IR spectrophotometer (Shimadzu). The scanning wavenumber ranged from 4,000 to 400 cm^{-1} and the spectral resolution was 4 cm^{-1} .

Statistical analysis

Mean of three replications was considered as the final reading for all the analysis. Data were analyzed using analysis of variance (ANOVA) using Prism6 software (GraphPad Inc.). The significant difference ($P \leq 0.001$) was calculated.

Results

Isolation and identification of VITJzN04

Microorganisms associated with rhizospheric soil are considered efficient agents to remediate pesticide (Elcey and Kunhi 2010). In this study, highly efficient indigenous yeast strain was obtained from sugarcane fields by enrichment culture, which was capable of utilizing lindane as a sole carbon and energy source in mineral medium. The yeast strain VITJzN04 was identified using a polyphasic approach, combining morphological, physiological and genetic identity and characters. The yeast strain when cultured on YEPD agar plates for 48 h (Fig. 1a), formed

colonies approximately 5 mm diameter, which appeared circular, convex, creamy white colour with an entire margin. The microscopic images of VITJzN04 under bright field and atomic force microscopes (AFM) are given in the Fig. 1b, c respectively.

The biochemical characterization was used to identify the isolate to genus level, which was performed by rapid identification using a Vitek-2 compact yeast card reader. The results of rapid biochemical test (Supplement Table 1) revealed that the lindane degrading yeast showed maximum identity with genus *Candida* with a confidence level of 99 %.

The sequence and phylogenetic analysis of the strain VITJzN04 were performed by neighbour-joining algorithm as shown in Fig. 2. The partial rDNA sequence (1,415 bp) was matched with those published in the NCBI database, and close relatedness was seen with the genus *Candida*. Therefore, the isolated yeast strain was named as *Candida* sp. VITJzN04 and accessible in the international culture maintenance MCC in NCCS, Pune, India.

Degradation characteristics of lindane by VITJzN04

Growth kinetics

In the growth experiments, strain VITJzN04 grew well, utilizing lindane as the sole carbon and energy source (Fig. 3a). When grown in an initial concentration of 100 mg L^{-1} , the complete utilization of lindane was noted within 3rd of incubation and the culture approached stationary phase on the 4th day. A maximum biomass of 1.3 g L^{-1} was marked in this case. The simultaneous chloride release into the medium was examined and significant correlation between chloride release, lindane degradation and biomass yield was noted.

Optimization of the lindane degrading conditions by strain VITJzN04

The optimum range of the five significant factors: pH 3–8 (Fig. 3b); temperature 20–40 °C (Fig. 3c); inoculum dosage 0.02–0.1 g L^{-1} (Fig. 3d) and lindane concentration 200–1,000 mg L^{-1} (Fig. 3e) affecting degradation were determined in separate experiments. The growth and utilization of lindane by VITJzN04 was found greatly influenced by the tested factors. Optimum pH for lindane degradation by VITJzN04 was found as 7. At pH below 5 and above 7, growth of yeast strain was found to be low. At an incubation temperature of 30 °C, initial lindane concentration was reduced by 92 %, therefore this temperature was considered as optimum. The optimum dosage of inoculum of VITJzN04 for the degradation of 100 mg L^{-1} was found as 0.06 g L^{-1} . At this dosage, the degradation

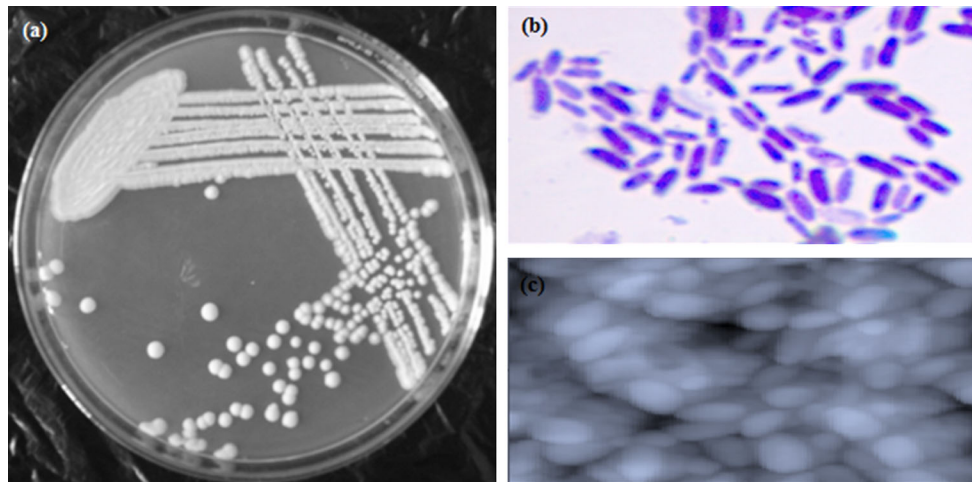
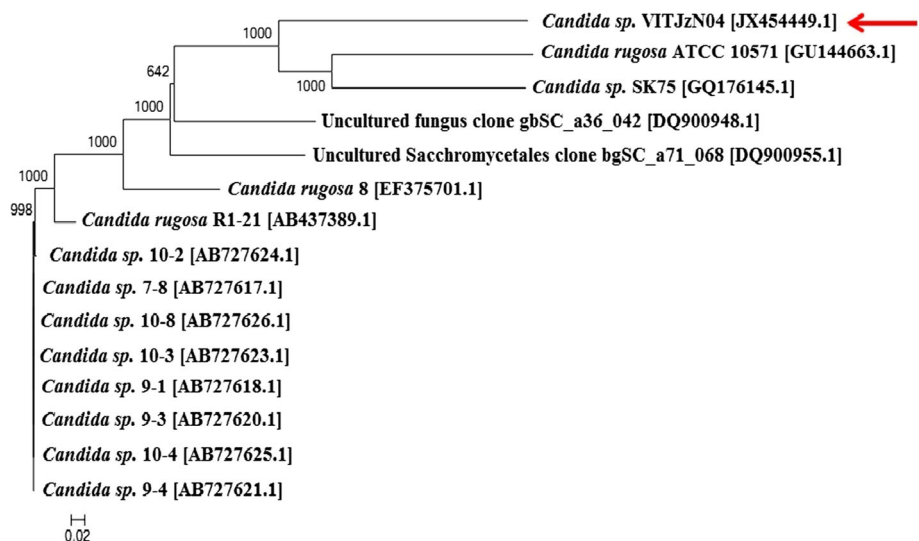


Fig. 1 Morphological characteristics of strain VITJzN04 **a** on YEPD agar after 48 h, **b** under bright field microscope ($\times 100$) and **c** under atomic force microscope ($\times 25 \mu\text{m}$)

Fig. 2 Phylogenetic analysis of VITJzN04 and related species by the neighbor-joining approach. The Genbank accession numbers are included in the *parentheses*. Boot strap values are given at branch node. The *scale bars* represent 0.05 substitutions per site



process reached equilibrium within 2 days. In the present study, VITJzN04 showed great efficacy of degrading lindane until an initial concentration 600 mg L^{-1} in 6 days, after which the degradation efficiency decreased significantly with the increase in concentration. Therefore, initial concentration of 600 mg L^{-1} was the optimized concentration of lindane.

Kinetic studies of lindane degradation by VITJzN04

The degradation of lindane (600 mg L^{-1}) was fitted with the four mathematical kinetic models as shown in Fig. 4a–d. The results show that the degradation kinetics of lindane by VITJzN04 can be described well by both first-order and pseudo-first-order reaction kinetics with respect to the aqueous concentration of lindane. The apparent kinetic rate constants (K), half-lives ($T_{1/2}$) and the regression equations under given conditions for each reaction model are

presented in Table 2. The regression coefficient, R^2 is highest (0.954) in both the first-order and pseudo-first order kinetic model, indicating that the degradation kinetics of lindane by VITJzN04 follows first-order and pseudo-first-order reactions well. This implies that the removal of lindane by VITJzN04 is a time dependant process. The calculated degradation rate constant K is 0.588 day^{-1} and the theoretical half-life of lindane is 1.17 days.

Effect of H_2O_2 in the enhancement of lindane degradation

The effect of H_2O_2 at various concentrations on enhancement of lindane degradation by VITJzN04 was studied. As seen in Fig. 4e, within the range of $0\text{--}10 \text{ mmol L}^{-1}$, H_2O_2 exhibited significant promotion of lindane degradation by the yeast strain. About 3–12 % lindane was oxidized when the concentration of H_2O_2 was increased from 1 to

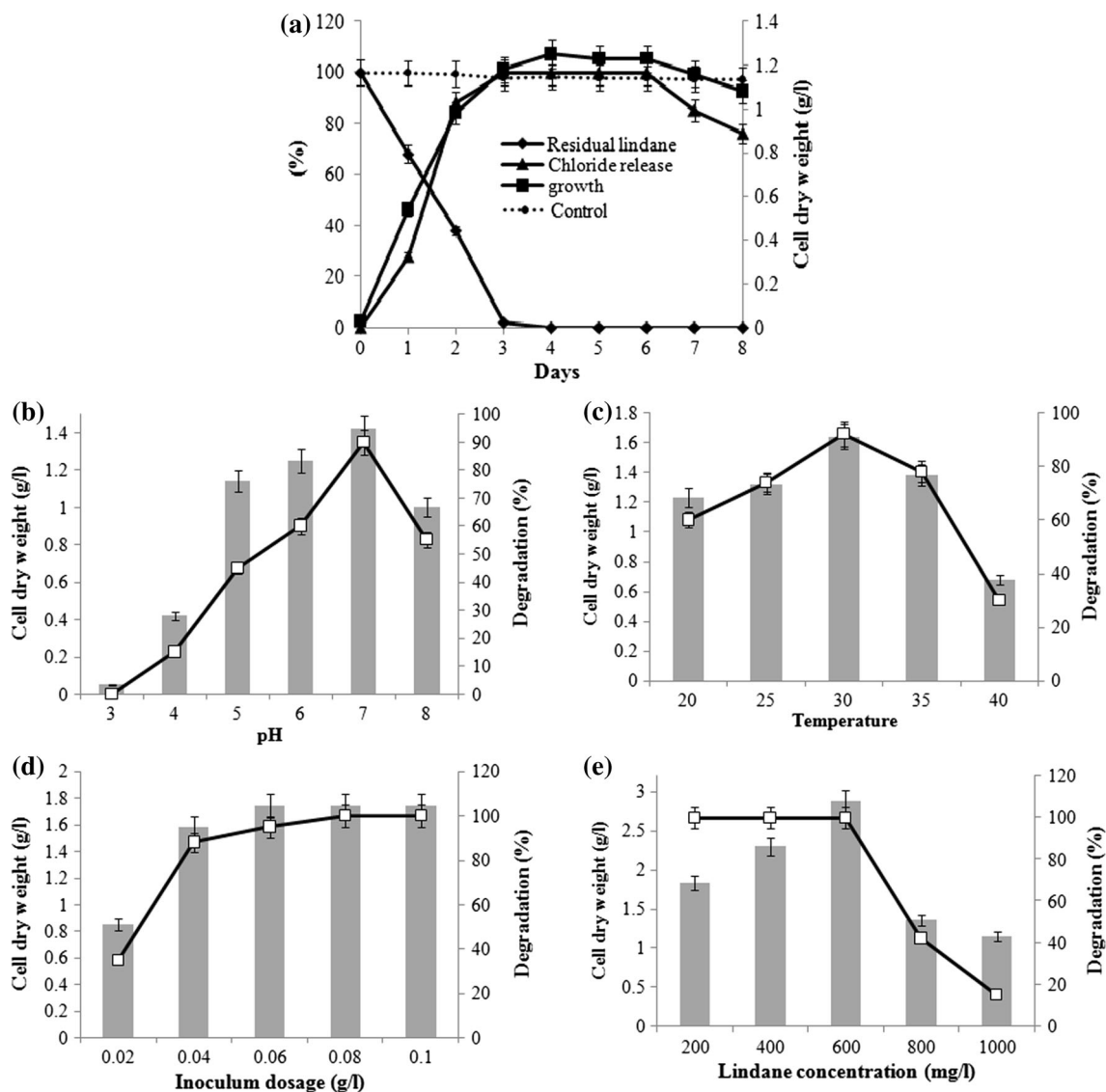


Fig. 3 a Growth of VITJzN04 in MM with lindane (100 mg L^{-1}) corresponding to lindane degradation and chloride ion release; Optimization of growth parameters b pH, c temperature, d inoculum dosage and e lindane concentration. The bar graphs represent the cell

dry weight and line graphs represent the lindane degradation. Error bars on the curves represent standard deviation of triplicate samples. $*P \leq 0.0001$

12 mmol L^{-1} in the medium without yeast culture. The addition of H_2O_2 in the culture medium of VITJzN04 enhanced the removal of lindane from 60 to 92 % on the third day of incubation.

Elucidation of lindane degradation pathway

Intermediates of lindane identified by GC–MS measurement

As shown in Fig. 5a, GC–MS analysis of lindane and its products after complete degradation identified six possible metabolites using an NIST library identification program. Lindane, which eluted with a retention time of 15.7 min, was

gradually degraded into infinitesimal quantities. Sample withdrawn on the day 2, indicated the presence of 2 possible new intermediate peak that eluted at 21.3 and 24.06 min was captured and identified as γ -pentachlorocyclohexane (γ -PCCH) and trichloro-2,5-cyclohexadiene-1-ol (TCCH-1-ol), both of which was detected on day 4 as well, indicating that they were formed during the initial phase of degradation and was then transformed into other compounds for further degradation. Another three apparent metabolites that were eluted at 18.59, 14.02 and 9.12 min were seen on 4th day of incubation and was confirmed to be 2,5-Dichlorohydroquinone (2,5-DCHQ), 2-Chlorohydroquinone (2-CHQ), Hydroxymuconicsemialdehyde (HMSA), in the order, according to the NIST library. On the 6th day, a new product eluted at 8.06 min, identified as

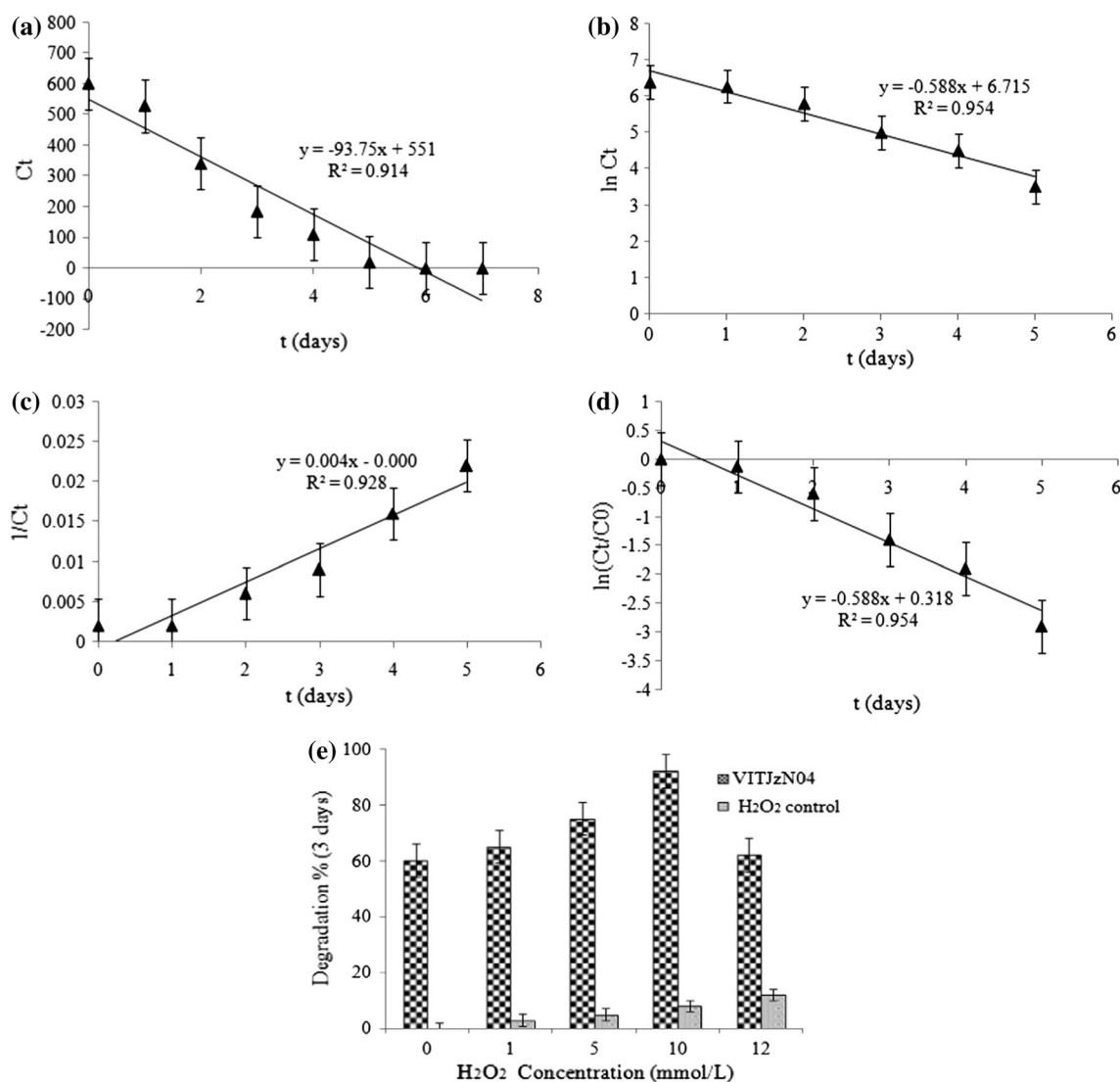


Fig. 4 Kinetic Plot of **a** zero order; **b** first-order; **c** second-order and **d** pseudo-first order reaction model for lindane degradation by VITJzN04. **e** Effect of H_2O_2 on degradation of 600 mg L^{-1} lindane by VITJzN03 at 30°C for 3 days ($*P \leq 0.0001$)

maleyl acetate (MA) appeared in chromatogram, simultaneously with the absence of parent lindane peak, indicating complete transformation of lindane into metabolites by VITJzN04. Eventually, no persistent accumulative product was detected by GC–MS analysis and base peak was detected on the final day of assay. The mass spectra of the lindane and degraded products are given in Table 3.

FTIR spectra

FTIR spectra of control lindane (Fig. 5b) showed the specific peaks in CH stretch (above $3,000 \text{ cm}^{-1}$), C–C skeletal vibrations ($1,350\text{--}1,000 \text{ cm}^{-1}$), CH–Cl stretch ($800\text{--}700 \text{ cm}^{-1}$) and benzene rings corresponding to the peak at $1,650\text{--}1,600 \text{ cm}^{-1}$. The second spectra illustrated, degraded products of lindane on day 3 showed peak at

$3,600 \text{ cm}^{-1}$ for hydroxyl group for aromatic primary alcohols representing hydroxyl derivatives of chlorocyclohexadienes; peak at $1,680 \text{ cm}^{-1}$ for quinone stretch supporting the formation hydroquinones; peak at wave number $1,300\text{--}1,400 \text{ cm}^{-1}$ indicated aldehyde stretches and benzene rings. Peak around $800\text{--}600 \text{ cm}^{-1}$ indicated the presence of C–Cl stretch.

Proposed lindane degradation pathway by strain VITJzN04

From the number of aromatics identified by the detection methods discussed above, a possible degradation pathway is proposed (Fig. 6). In other words, the parent lindane was first dechlorinated to form, penta-chloro and subsequently the tetra-chloro derivative. Further by multi step reduction and dechlorination, 2,5-DCHQ is formed which is reduced

Table 2 The kinetic parameters for the degradation of lindane at 600 mg L⁻¹ by VITJzN04

Kinetic model	Parameters	<i>Candida</i> sp. VITJzN04
Zero order	Regression equation	$C_t = -93.75t + 551$
$C_t - C_0 = Kt$	K (day ⁻¹)	93.75
$T_{1/2} = \frac{C_0}{2K_0}$	T _{1/2}	3.2
	R ²	0.914
First order	Regression equation	$\ln C_t = -0.588t + 6.715$
$\ln C_t = K_1t + \ln C_0$	K (day ⁻¹)	0.588
$T_{1/2} = \frac{\ln 2}{K_1}$	T _{1/2}	1.17
	R ²	0.954
Second order	Regression equation	$1/C_t = 0.001t - 0.00$
$\frac{1}{C_t} = \frac{1}{C_0} + K_2t$	K (day ⁻¹)	0.001
$T_{1/2} = \frac{C_0}{2K_2}$	T _{1/2}	75
	R ²	0.932
Pseudo-first order	Regression equation	$C_t = 0.318 e^{-0.588t}$
$C_t = C_0 \cdot e^{-Kt}$	K (day ⁻¹)	0.588
$T_{1/2} = \frac{\ln 0.5}{-K}$	T _{1/2}	1.17
	R ²	0.954

R² = regression coefficient; K = degradation rate constant; T_{1/2} = half life period

to 2-CHQ. The closed ring is then opened and hydrolysed to produce HMSA. HMSA is oxidized to form maleyl acetate which would be incorporated directly into cellular tricarboxylic acid cycle for generation of energy. Some undetected intermediates such as dichlorocyclohexa-2,5-diene-1,4-diol (DCCH-2,5-diene-1,4-diol) and hydroquinone (HQ) are shown within dotted-lines. It is supposed that these compounds may have accumulated to trace amounts and was spontaneously transformed. The existence of these transient metabolites can be inferred from subsequent compounds detected. Similar degradation route has been observed in a bacterial strain *Sphingomonas japonica* UT26 (Nagata et al. 2007).

Enzymatic activities during lindane degradation

Table 4 represents the enzyme activities in the culture supernatant and cell lysate on the 2nd, 4th and 6th day of lindane degradation by VITJzN04. On the initial phase of lindane breakdown, lindane dechlorinase had profound activity both in the supernatant and the cell lysate whereas the reverse trend was observed for other degradative enzymes studied. Lindane dechlorinase and dehalogenase activities reduced with time, as the chlorinated compounds

seemed to reduce with time. The DCHQ reductive dechlorinase activity was negligible on the day 2, but was found to be increased on the 4th day and further decreased over incubation time. Similar activity was seen in case of other enzymes such as Mn peroxidase and lignin peroxidase. The activity of lindane dechlorinase, dehalogenase and peroxidases was more in cell supernatant than in the cell lysate which implicated the extracellular nature of these enzymes.

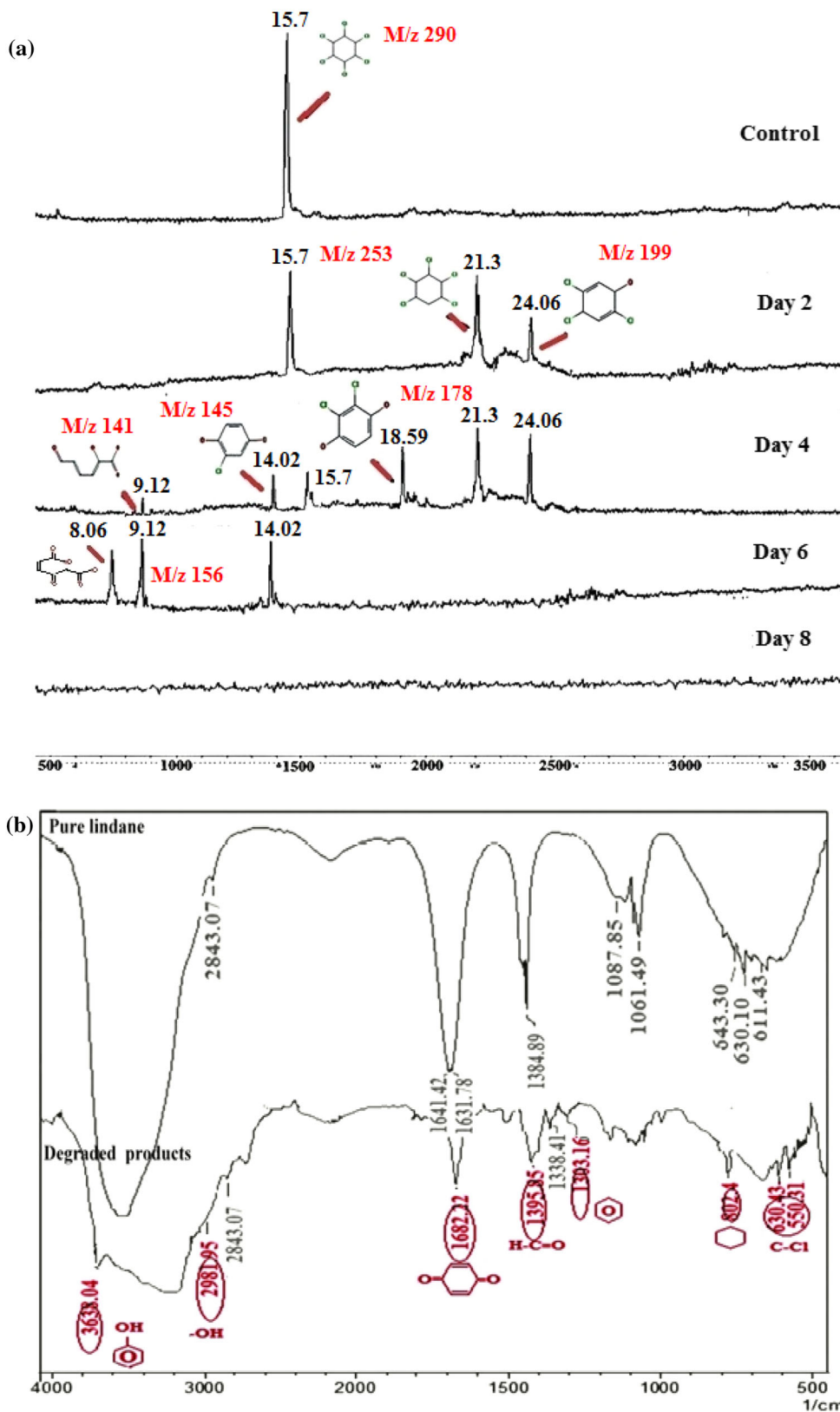
Discussion

Lindane, a broad spectrum synthetic organochlorine pesticide, has been heavily used throughout the world for pest control both in agriculture and medicine during the past few decades, resulting in widespread contamination. Bioremediation employs the use of living microorganisms to degrade and detoxify pollutants, and is generally implied to be most significant route determining the fate and behaviour of xenobiotics in the environment (Singh 2009). Several microorganisms capable of degrading lindane have been isolated from diverse geographic locations. Yeast possesses the biochemical and ecological capacity to degrade environmental organic chemicals, either by chemical modification or by influencing chemical bioavailability (Singh 2009). But the potential use for yeast in bioremediation of lindane and other pesticides has not received the attention it deserves. This report is a complete elucidation of the mechanism of lindane degradation by the yeast species.

In the present study, isolation of lindane-degrading yeast by enrichment culture from sugarcane fields, allowed us to obtain a very potent and fast growing yeast strain, VIT-JzN04, which was identified as *Candida* sp. based on morphological and 18S rDNA gene analysis. Previous research has shown that the potential lindane-degrading microorganism were mostly from genera *Pseudomonas*, *Sphingomonas*, *Streptomyces*, and *Fusarium* (Salam et al. 2013), while *Candida* sp. VITJzN04 appeared to be a new species that was found greatly effective in degrading lindane and its metabolites. Members of this genus are ubiquitous in the environment and some isolates have antagonistic effect on pathogen due to their production of bioactive secondary metabolites (Bello et al. 2008). However, very little knowledge is available regarding the use of these yeast to degrade and detoxify pollutants. Therefore, the current findings expand their use.

Candida sp. VITJzN04 utilized lindane as sole carbon and energy source for growth in MM. During the incubation the yeast in MM with lindane, the organism attained exponential phase on the day 1 itself, furthermore complete

Fig. 5 a GC–MS analysis of lindane and its degradation products at 2, 4, 6 and 8 days; **b** FTIR analysis of lindane before (pure lindane) and after degradation by strain VITJzN04 for 3 days

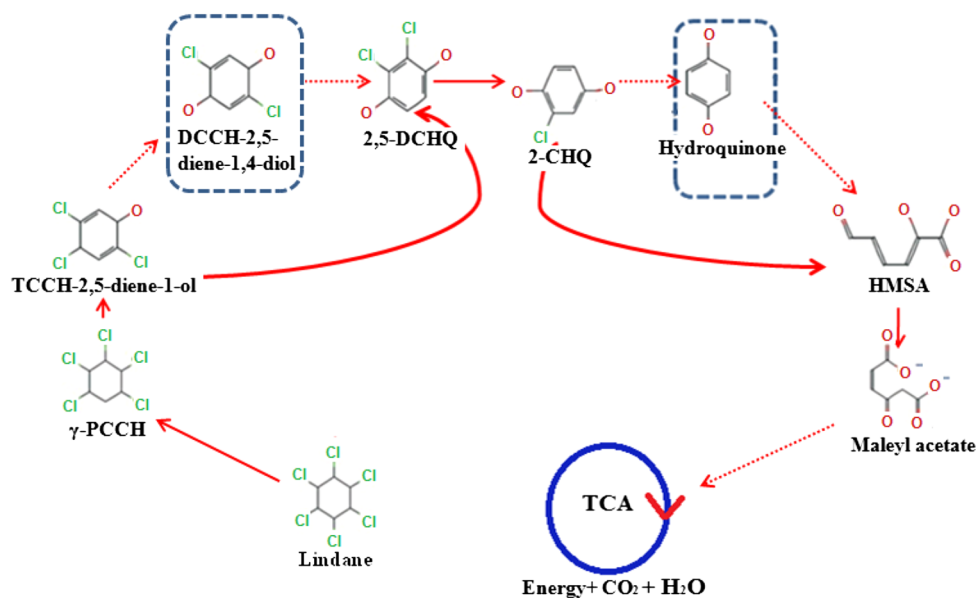


removal of lindane was noted at the end of stationary phase. This was complemented with the 100 % release of chloride ions from the lindane, which is clear indication of

complete and rapid mineralization. This observation is extremely important from the perspective of environmental pollution control.

Table 3 GC retention times and mass spectra of lindane and the various metabolites produced by VITJzN04

Days	GC-Rt (min)	Mass spectrum m/z (relative intensity)	Suggested metabolite
Control	15.7	290(1), 254(5), 223(5), 217(80), 216(5), 185(25), 181(100), 156(10), 147(20), 113(15), 111(100), 96(5), 85(40), 73(10), 51(40)	Lindane
2 days	21.3	253(4), 224(5), 182(7), 168(9), 140(12), 126(15), 112(22), 99(33), 85(100), 57(100)	γ -pentachlorocyclohexane (γ -PCCH)
	24.06	199(2), 180(4), 146(85), 128(10), 111(30), 99(20), 89(5), 81(15), 75(22), 51(11)	Trichloro-2,5-cyclohexadiene-1-ol (TCCH-1-ol)
4 days	18.59	178(45), 176(10), 150(40), 141(5), 120(50), 113(60), 90(3), 88(100), 62(20), 60(100), 53(60), 50(25), 37(5), 25(10)	2,5-dichlorohydroquinone (2,5-DCHQ)
	14.02	145(100), 95(5), 80(30), 77(3), 64(5), 51(50), 37(15), 25(4)	2-chlorohydroquinone (2-CHQ)
	9.12	141(100), 139(18), 131(10), 125(25), 113(5), 106(10), 91(5)	Hydroxymuconicsemialdehyde (HMSA)
6 days	8.06	156(50), 148(46), 145(85), 135(25), 131(7), 125(8), 123(37), 121(55), 115(4), 111(100)	Maleyl acetate (MA)

**Fig. 6** The proposed lindane degradation pathway by strain VITJzN04. The complete *arrows* represent products identified by GC–MS. The *dotted arrows* represent transient metabolites, which were not detected in GC–MS**Table 4** Enzyme activities (U/mL) in the culture supernatant and cell free extracts of VITJzN04

Enzymes	Day 2		Day 4		Day 6	
	A	B	A	B	A	B
Lindane dechlorinase	9.45 ± 0.05	3.42 ± 0.05	7.25 ± 0.05	3.42 ± 0.05	1.52 ± 0.05	1.94 ± 0.05
Lindane dehalogenase	2.42 ± 0.05	1.5 ± 0.05	3.45 ± 0.05	1.2 ± .05	0.84 ± 0.05	0.45 ± 0.05
DCHQ reductive dechlorinase	–	0.24 ± 0.43	0.98 ± 0.25	2.6 ± 0.28	0.45 ± 0.43	1.1 ± 0.43
Laccase	–	–	–	–	–	–
Lignin peroxidase	3.42 ± 0.24	2.32 ± 0.73	5.86 ± 0.66	3.41 ± 0.05	3.5 ± 0.21	1.6 ± 0.07
Manganese peroxidase	0.72 ± 0.03	0.34 ± 0.03	1.26 ± 0.03	0.72 ± 0.03	0.55 ± 0.03	0.08 ± 0.03

A-Enzymes activity in the culture supernatant; B-Enzyme activity in the cell lysate

In our previous study with yeast *Rhodotorula* VITJzN03, the optimum pH for degradation of lindane was found to be 6.0 (Salam et al. 2013). Other workers reported that a neutral range of pH 6–8 was most favourable for lindane degradation (Elcey and Kunhi 2010). In the case of incubation temperature, the behaviour of *Candida* sp. VITJzN04 was similar to the results obtained in other studies (Zhang et al. 2010, 2012; Salam et al. 2013) all of which documented 30 °C as optimum for lindane degradation. However, the fungal degradation of lindane is also reported at lower temperatures (Rigas et al. 2007; Dritsa et al. 2009). Guillen-Jimenez et al. (2012) described that the use of larger inoculum size increased the degradation efficiency and decreased the time of degradation of highly toxic compounds. The elevation of microbial load will increase the microbial activity and thus the number metabolic pathways involved. Increasing the inoculum size from 0.02 to 0.06 mg L⁻¹ promoted the degradation efficiency from 40 to 100 % for the same incubation time. When the initial inoculum dosage was increased further, there was no significant difference in the degradation rate. Similar effect on lindane degradation upon increased inoculum size was noted in other studies as well (Chaudhary et al. 2006). The concentration of the target pollutant is an important factor which affects the microbial growth as well as the rate of degradation. Low concentration of pollutant might not be able to induce the enzymes of degradative activity while high levels may be toxic to the microorganisms (Awasthi et al. 2000). It was noteworthy that this particular strain could tolerate till a high concentration of 1,000 mg L⁻¹ degrading about 42 % of lindane. However, the specific degradation rate decreased with increase in initial lindane concentration. These observations indicated that increased lindane concentration had a profound effect on degradation performance of strain VITJzN04, but did not lead to complete inhibition. The other yeast strain, *Rhodotorula* sp. VITJzN03, already reported as the best known lindane degrader completed degradation of 600 mg L⁻¹ lindane within 8 days. Therefore, the presently isolated yeast *Candida* sp. VITJzN04 proved higher potentiality on lindane degradation making it the most efficient agent of all the lindane degraders so far studied.

Hydrogen peroxide is known to facilitate the degradation of chemical pollutants especially persistent organic pollutants (Svrcek et al. 2010). In the present study, addition of H₂O₂ into the MM facilitated the lindane dechlorination by *Candida* sp. VITJzN04. It might be possibly due to increased availability of electron donor for the removal of Cl⁻ and oxidation of lindane. Similar response on the use of H₂O₂ to facilitate the degradation of other organic pollutants was reported by other workers (Svrcek et al. 2010; Ye et al. 2013).

The kinetic analysis of lindane degradation by the yeast *Candida* sp. VITJzN04 was done by comparing, with zero-

order, first-order, pseudo-first order and second order kinetic equations. It was found that the degradation of lindane was dependant on the substrate concentration, which was well explained by first order equation. Previously, the degradation kinetic of lindane was also studied in other organisms, where the calculated half-life of lindane were described as follows—1.66 days for *Rhodotorula* sp. VITJzN03 (Salam et al. 2013); 4.78 days for *Anabeana azotica* (Zhang et al. 2012). Clearly, the calculated half-life of lindane, in the present study is shortened (1.17 days) than in the earlier findings indicating the astonishing potency of the strain in lindane degradation.

Previous reports have proved the ability of microbial consortium to supersede the ability of individual microorganisms in biodegradation and remediation of toxic waste from contaminated soil as well as industrial effluents (Pant and Adholeya 2010). In the present study, the yeast strain VITJzN04 was found to attain complete degradation of high concentration of lindane within a period of 6 days, marking its outstanding potentiality in lindane remediation. The performance of the organism may be enhanced by providing a microbial consortium for lindane degradation in aqueous and soil environments.

GC–MS-based metabolite analysis has profound implications for discovering the mode of action of pesticides and helps to unravel the effect of altered gene expression on metabolism and organism performance in biotechnological applications (Du et al. 2013). This technique is irreplaceable because of its unique ability to detect compounds with high volatilities, low boiling temperatures and low molecular weights with high sensitivity. In our study, the metabolites formed during the degradation of lindane by *Candida* sp. VITJzN04 was identified by GC–MS. In our earlier report on lindane degradation by yeast *Rhodotorula* sp. VITJzN03, chlorobenzene derivatives were detected during the lindane degradation. In the present study, the presence of metabolites such as derivatives of hydroquinone and HMSA signifies that different enzymatic mechanism for lindane degradation exist and can lead to the differential accumulation of compounds. The major metabolites reported for fungi in the existing papers during lindane degradation are γ -PCCH and benzoic acid derivatives (Guillén-Jiménez et al. 2012).

Although, several fungi capable of degrading lindane have been isolated and studied, yet no proper report on the enzymatic mechanism of degradation is available. A possible reason attributed to this might be the involvement of complex and nonspecific enzyme systems, due to high degree of chlorination in lindane molecule. Oxidative enzymes and cytochrome p450 enzymes have been reported to be responsible for pesticide degradation in eukaryotes (Van Eerd et al. 2003). Recent studies have focused mostly on the activities of the enzymes viz. laccase, Mn

peroxidase and lignin peroxidase to understand the role of fungi in lindane degradation (Nagpal et al. 2008; Guillén-Jiménez et al. 2012). On the other hand, lin-operon, a set of enzymes holds responsible for bacterial degradation of lindane (Lal et al. 2010). In the present study, activities of both fungal and bacterial enzymes were tested in lindane containing cultures. Significant enzyme activities viz. Mn-peroxidase and lignin peroxidase along with lindane dechlorinase, lindane dehalogenase and reductive dechlorinase was noted over the incubation period. In contrast, laccase activities were not shown in the cultures. This is the first report on the involvement of the enzymes viz. lindane dechlorinase and lindane dehalogenase during lindane degradation in yeast species.

Lignolytic enzymes namely lignin peroxidase, laccase and manganese peroxidases were found to play important role in organic pollutant remediation (Pant and Adholeya 2009a, b). Our research findings can enhance the understanding of the role of yeasts towards enzyme mediated remediation of lindane. Solid state fermentation of the yeast *Candida* sp. VITJzN04 can improve the production of enzymes which can be used for enzyme technologies and remediation purposes (Pant and Adholeya 2009b).

Conclusions

In the present study, *Candida* VITJzN04 obtained from sugarcane field exhibited greater potential for complete degradation of 600 mg L⁻¹ lindane and its hydrolytic metabolites within 6 days in aqueous medium. The kinetic studies revealed a very good compliance with the first and pseudo first order models. Enzyme analysis indicated the involvement of lindane dechlorinase, lindane dehalogenase, DCHQ reductive dechlorinase, Mn peroxidase and lignin peroxidase enzymes during lindane degradation by the yeast strain. Based on our results, possible hypothetical degradation route is proposed. To our knowledge, this is the first report on the detailed biodegradation mechanism of lindane in yeast. It can be concluded that the yeast strain *Candida* sp. VITJzN04 may serve as a potential bioresource for efficient decontamination of lindane polluted environment.

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References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 5:403–410
- Alvarez A, Yanez ML, Benimeli CS, Amoroso MJ (2012) Maize plants (*Zea mays*) root exudates enhance lindane removal by native *Streptomyces* strains. *Int Biodeter Biodegr* 66:14–18
- Awasthi N, Ahuja R, Kumar A (2000) Factors influencing the degradation of soil applied endosulfan isomers. *Soil Biol Biochem* 32:1697–1705
- Bello GD, Monaco C, Rollan MC, Lampugnani G, Arteta N et al (2008) Biocontrol of postharvest grey mould on tomato by yeasts. *J Phytopathol* 156:257–263
- Bergmann JG, Sanik J (1957) Determination of trace amounts of chlorine in naphtha. *Anal Chem* 29:241–243
- Beyer A, Matthies M (2001) Long-range transport potential of semi-volatile organic chemicals in coupled air–water systems. *Environ Sci Pollut Res* 8(3):173–179
- Capellos C, Bielski BH (1972) Kinetic systems: mathematical description of chemical kinetics in solution. Wiley-Inter science, New York
- Chaudhary P, Kumar M, Khangarot BS, Kumar A (2006) Degradation and detoxification of hexachlorocyclohexane isomers by *Pseudomonas aeruginosa* ITRC-5. *Int Biodeter Biodegr* 57:107–113
- De Paolis MR, Lippi D, Guerriero E, Polcaro CM, Donati E (2013) Biodegradation of α -, β -, and γ -hexachlorocyclohexane by *Arthrobacter fluorescens* and *Arthrobacter giacomelloi*. *Appl Biochem Biotechnol*. doi:10.1007/s12010-013-0147-9
- Dritsa V, Rigas V, Doulia D, Avramides EJ, Hatzianestis I (2009) Optimization of culture conditions for the biodegradation of lindane by the polypore fungus *Ganoderma australe*. *Water Air Soil Poll* 204(1):19–27
- Du L, Zhao M, Li G, Xu F, Chen W, Zhao Y (2013) Biodegradation of malachite green by *Micrococcus* sp. strain BD15: biodegradation pathway and enzyme analysis. *Int Biodeter Biodegr* 78:108–116
- Dykaar BB, Kitanidis PK (1996) Macrotransport of a biologically reacting solute through porous media. *Water Resour Res* 32:307–320
- Elcey CD, Kunhi AAM (2010) Substantially enhanced degradation of hexachlorocyclohexane isomers by a microbial consortium on acclimation. *J Agric Food Chem* 58:1046–1054
- Fuentes MS, Sáez JM, Benimeli CS, Amoroso MJ (2011) Lindane biodegradation by defined consortia of indigenous *Streptomyces* strains. *Water Air Soil Poll* 222:217–231
- Guillén-Jiménez F, Cristiani-Urbina E, Cancino-Díaz JC, Flores-Moreno JL, Barragán-Huertaa BE (2012) Lindane biodegradation by the *Fusarium verticillioides* AT-100 strain, isolated from *Agave tequilana* leaves: kinetic study and identification of metabolites. *Int Biodeter Biodegr* 74:36–47
- Herrero-Mercado M, Waliszewski SM, Valencia-Quintana R, Caba M, Hernández-Chalate F, García-Aguilar E, Villalba R (2010) Organochlorine pesticide levels in adipose tissue of pregnant women in Veracruz, Mexico. *Bull Environ Contam Toxicol* 4(6):652–656
- Husaini A, Fisol FA, Yun LC, Hussain MH, Roslan HA (2011) Lignocellulolytic enzymes produced by tropical white rot fungi during biopulping of *Acacia mangium* wood chips. *J Biochem Technol* 3(2):245–250
- Kidd P, Prieto-Fernández A, Monterroso C, Acea MJ (2008) Rhizosphere microbial community and hexachlorocyclohexane degradative potential in contrasting plant species. *Plant Soil* 302:233–247
- Lal R, Pandey G, Sharma P, Kumari K, Malhotra S, Rinku Pandey, Raina V, Kohler HPE, Holliger C, Colin Jackson, Oakeshott JG (2010) Biochemistry of microbial degradation of hexachlorocyclohexane and prospects for bioremediation. *Microbiol Mol Biol R* 74:58–80
- Manickam N, Misra R, Mayilraj S (2007) A novel pathway for the biodegradation of γ -hexachlorocyclohexane by a *Xanthomonas* strain ICH12. *J Appl Microbiol* 102:1468–1478

- Miyauchi K, Suh SK, Nagata Y, Takagi M (1998) Cloning and sequencing of a 2,5-dichlorohydroquinone reductive dehalogenase gene whose product is involved in degradation of gamma-hexachlorocyclohexane by *Sphingomonas paucimobilis*. *J Bacteriol* 180(6):1354–1359
- More SS, P S R, K P, M S, Malini S, S M V (2011) Isolation, purification, and characterization of fungal laccase from *Pleurotus* sp. *Enzyme Res* 2011: 248735. doi:10.4061/2011/248735
- Nagata Y, Endo R, Ito M, Ohtsubo Y, Tsuda M (2007) Aerobic degradation of lindane (γ -hexachlorocyclohexane) in bacteria and its biochemical and molecular basis. *Appl Microbiol Biotechnol* 76:741–752
- Nagpal V, Srinivasan MC, Paknikar KM (2008) Biodegradation of γ -hexachlorocyclohexane (lindane) by a non- white rot fungus *Conidiobolus* 03-1-56 isolated from litter. *Indian J Microbiol* 48:134–141
- Pant D, Adholeya A (2009a) Nitrogen removal from biomethanated spentwash using hydroponic treatment followed by fungal decolorization. *Environ Eng Sci* 26(5):559–565
- Pant D, Adholeya A (2009b) Concentration of fungal ligninolytic enzymes produced during solid-state fermentation by ultrafiltration. *World J Microbiol Biotechnol* 25:1793–1800
- Pant D, Adholeya A (2010) Development of a novel fungal consortium for the treatment of molasses distillery wastewater. *Environmentalist* 30:178–182
- Petrucci RH, Harwood WS, Herring G, Madura JD (2007) General chemistry: principles and modern applications, 9th edn. Pearson Education, Upper Saddle River
- Phillips TM, Seech AG, Lee H, Trevors JT (2005) Biodegradation of hexachlorocyclohexane (HCH) by microorganisms. *Biodegradation* 16:363–392
- Pinero GM, Izquierdo CP, Allara CM, García UA (2007) Residuos de plaguicidas organoclorados en 4 tipos de aceites vegetales. *Arch Latinoam Nutr* 57:397–401
- Rigas F, Papadopoulou K, Dritsa V, Doulia D (2007) Bioremediation of a soil contaminated by lindane utilizing the fungus *Ganoderma australe* via response surface methodology. *J Hazard Mater* 140(1–2):325–332
- Sagar V, Singh DP (2011) Biodegradation of Lindane pesticide by non white-rots soil fungus *Fusarium* sp. *World J Microbiol Biotechnol* 27:1747–1754
- Salam JA, Lakshmi V, Das D, Das N (2013) Biodegradation of lindane using a novel yeast strain, *Rhodotorula* sp. VITJzN03 isolated from agricultural soil. *World J Microbiol Biotechnol* 29(3):475–487
- Singh BK (2009) Organophosphorus-degrading bacteria: ecology and industrial applications. *Nat Rev Microbiol* 7:156–163
- Svrcek J, Marhoul A, Kacer P, Kuzma M, Pánek L, Cervený L (2010) The influence of operating conditions on the efficiency of vapour phase hydrogen peroxide in the degradation of 4-(dimethyl-amino)benzaldehyde. *Chemosphere* 81(5):617–625
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Van Eerd LL, Hoagland RE, Hall JC (2003) Pesticide metabolism in plants and microorganisms. *Weed Sci* 51:472–495
- Ye J, Yin H, Peng H, Bai J, Xie D, Wang L (2013) Biosorption and biodegradation of triphenyltin by *Brevibacillus brevis*. *Bioresource Technol* 129:236–241
- Zhang H, Yang C, Zhao Q, Qiao C (2009) Development of an autofluorescent organophosphates-degrading *Stenotrophomonas* sp. with dehalogenase activity for the biodegradation of hexachlorocyclohexane (HCH). *Bioresource Technol* 100:3199–3204
- Zhang H, Wan H, Song L, Jiang H, Wang H, Qiao C (2010) Development of an auto fluorescent *Pseudomonas nitroreducens* with dehydrochlorinase activity for efficient mineralization of gamma-hexachlorocyclohexane (gamma-HCH). *J Biotechnol* 146(3):114–119
- Zhang H, Hu C, Jia X, Xu Y, Wu C, Chen L, Wang F (2012) Characteristics of γ -hexachlorocyclohexane biodegradation by a nitrogen-fixing cyanobacterium. *Anabaena azotica* *J Appl Phycol* 24:221–225