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Bioremediation potential of newly isolated *Bacillus albus* strain VKDS9 for decolourization and detoxification of biomethanated distillery effluent and its metabolites characterization for environmental sustainability

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ABSTRACT

In India, the environment friendly and sustainable management of colored effluents emanating from alcohol-producing distilleries has become a serious environmental concern. The current study aimed to examine the efficiency of ligninolytic enzyme-producing bacterial strain isolated from ameliorated distillery sludge by enrichment method for its bioremediation and detoxification potential of biomethanated distillery effluent (BMDE). Out of nineteen bacterial isolates isolated from distillery sludge, one strain VKDS9, having higher ability towards the removal of effluent's color, was identified as *Bacillus albus* by 16S rRNA analysis. The bacterium showed significant decolourization of effluent up to 83% with subsequent reduction of biochemical oxygen demand (3261 mg/L), chemical oxygen demand (7870.5 mg/L), and other physico-chemical parameters including metallic and non-metallic elements in the presence of glucose (1% w/v) and peptone (0.5% w/v) at optimal temperature 37 °C, pH 8.0, and 180 rpm within 144 h. Further, biodegradation of effluent was confirmed by high performance liquid chromatography analysis that showed appearance/disappearance and reduction of several peaks. Moreover, GC–MS analysis of bacterial treated effluent displayed the disappearance of several chemical contaminants and formation of new metabolites which were formed as end products of biodegraded effluent. Furthermore, phytotoxicity of treated and untreated BMDE was evaluated using *Cicer arientinum* L. seeds revealed the biodetoxification of BMDE after treatment by *B. albus* strain VKDS9. This study concluded that *B. albus* strain VKDS9 can be used as an efficient biotechnological tool for

Abbreviations: BMDE, Biomethanated distillery effluent; BSTFA, N,O bis(trimethylsilyl)trifluoroacetamide; BOD, Biochemical oxygen demand; CA, Chromosomal aberrations; CFU, Colony-forming unit; COD, Chemical oxygen demand; CPCB, Central Pollution Control Board; EC, Electrical conductivity; EI, Electron ionization; FTIR, Fourier Transform Infrared Spectroscopy; GC–MS, Gas chromatography–mass spectrometry; GI, Germination index; HPLC, High performance liquid chromatography; HRT, Hydraulic retention time; LiP, Lignin peroxidase; MnP, Manganese peroxidase; MRPs, Maillard reaction products; NCBI, National Centre for Biotechnology Information; PI, Phytotoxicity index; Pt–Co, Platinum–cobalt; RRG, Relative root growth; RSG, Relative seed germination; RT, Retention time; SEM-EDX, Scanning Electron Microscope-Energy Dispersive X-ray; TN, Total nitrogen; TDS, Total dissolved solids; TMS, Trimethylsilyl; TOC, Total organic carbon; TSS, Total suspended solids; UV–Vis, Ultra violet–visible

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remediation of distillery effluent for its harmless dumping into the environment towards sustainable development.

1. Introduction

The fast-growing alcohol distilleries are considered as one of the significant environmental polluters in developing countries including India due to its production of significant volumes of complex and hazardous effluent that led to technical and techno-economical constraints in adoption of a eco-sustainable and safe disposal (Krishnamoorthy et al., 2019). According to All India Distiller's Association, about 397 distilleries are functioning in different states of India emanating an average of 3.5×10^{13} liters of alcohol and producing 40.9×10^{15} liters of spent wash annually (Kumar and Chandra, 2020). Several reports suggest that an average-sized molasses-based alcohol distillery generates about 12 to 15 liter of heavily polluted spent wash per liter of alcohol produced (Tripathi et al., 2021). Spent wash usually has strong color, extremely high COD, BOD, TOC, TDS, turbidity, and inorganic salts (Patyal, 2015; Shinde et al., 2020; Mikucka and Zielińska, 2020). The intense color of spent wash is primarily attributed to heterocyclic-nitrogenous, recalcitrant, acidic melanoidin pigment formed by browning reaction between reducing sugars and amino compounds (Ahmed et al., 2020). Moreover, spent wash has contained a substantial number of toxic substances including androgenic, genotoxic, and mutagenic chemicals including heavy metals (Kaushik et al., 2010; Chandra and Kumar, 2017a). The elevated amount of complex organic–inorganic and organometallic compounds makes these effluents persistent in water and soil, which are extremely toxic and recalcitrant to natural biodegradation (Fito et al., 2019).

In order to reduce the pollution load, currently, several distilleries are reutilizing the spent wash for generating methane as biofuel through anaerobic digestion (also called biomethanation) (Mohan et al., 2020). However, this anaerobic method is not enough for the efficient elimination of color and refractory chemicals from effluent. Consequently, distillery effluent converts darker after biomethanation due to complexation of lignin monomers, metallic elements, melanoidins, and other MRPs and treated spent wash still retain high color, BOD, and COD (Bhoite and Vaidya, 2018; Malik et al., 2019). The strong cations binding efficiency of melanoidin enhances its toxicity into the ecosystem and pose a serious threat to the health of aquatic and soil organisms (Alves et al., 2015; Hatano et al., 2016). Even though, several physico-chemical methods have been practiced for the efficient elimination and degradation of color pigment molecules from BMDE. These approaches have some drawbacks of greater use of expensive chemicals, low sensitivity, formation of excess sludge, and the plausible formation of secondary pollutants which also have secondary disposal problem (Rafigh and Rahimpour Soleymani, 2019; Ratna et al., 2021).

The serious devastating effects of colored distillery effluent on the aquatic ecosystem and organisms due to improper and indiscriminate dumping have been evidenced (Kumar and Gopal, 2001; Chauhan and Rai, 2010; Mahar et al., 2013). In spite of the harmful consequences, distilleries are being indiscriminately disposed their untreated or partly treated effluent to open lands and water bodies which not only deteriorates the health of the environment but also cause various human illnesses (Ramakritinan et al., 2005; Tripathy et al., 2020). Several refractory organic compounds, such as tetradecanoic acid, hexacosanoic acid, octadecanoic acid, β–sitosterol, and stigmasterol discharged in effluent are responsible for the chromosomal damage and alteration of α-amylase activity which leads to inhibition of germinating seeds (Kumar et al., 2021).

In the last few decades, innovative and environment friendly technology, such as bioremediation using potent organisms like bacteria, yeast, plants, fungi, algae, and actinomycetes to remediate or cleaning up many environmental pollutants in effluents has gained worldwide attention (Kidgell et al., 2014; USEPA, 2020; Ravikumar et al., 2021). The evolving significance of bacteria and possible role of their extracellular ligninolytic enzyme system to degrade or mineralize several recalcitrant and toxic pollutants emanating in distillery effluent has gained popularity in recent years (Kumar and Chandra, 2018; Chandra et al., 2018). However, the microbial growth and their enzyme activities in culture medium are affected by several nutritional and environmental factors. It is worth noticing that the remediation of distillery effluent by biological means usually requires enzymes to remain active under high levels of refractory organic, inorganic, and organometallic co-pollutants or extreme pH and temperature conditions. Hence, it is crucial to optimize nutritional and environmental factors process parameters and investigation of enzymes to achieve effective decolorization and detoxification of melanoidin to reduce the chemical load of spent wash discharged even after secondary treatment. Most of the scientific studies on the degradation and decolourization of diluted distillery effluent under optimization of two parameters, while there is very scanty information on decolourization and detoxification of BMDE using one-factor approach in static conditions (Santal et al., 2011, 2016). The investigation of toxicity of distillery effluent and the metabolites formed after biodegradation progression is one of the important issues in view of environmental sustainability (Malik et al., 2019; Agrawal et al., 2021).

Keeping in view of the above background, the aim of the study was to investigate the potential of a novel bacteria isolate, *Bacillus albus* strain VKDS9, that can decolourize and degrade toxic chemicals under optimized conditions in a batch process and enable its profitable application for remediation of BMDE at industrial scale. Further, the degraded metabolites

of BMDE were identified by various spectroscopic techniques. The reduction of toxicity of distillery effluent after bacterial degradation and decolourization was also investigated by using seeds of *Cicer arientinum* L. To our knowledge, *B. albus* is being reported first time for BMDE bioremediation process.

2. Material and methods

2.1. Reagents and chemicals

All the analytical grade chemicals, such as ethyl acetate, methanol, hydrogen peroxide, sodium sulfate, urea, ammonium nitrate, ammonium chloride, BSTFA, pyridine, guaiacol, and Azure-B were procured from Merck Life Science, India. Moreover, other reagents used for culture media preparation and optimization process like yeast extract, beef extract, peptone, sucrose, salts, and bacteriological agar were collected from Himedia Private Limited India, Maharashtra, India. Deionized water was used throughout the experiment. The healthy and uniform seeds of desi chickpea, *C. arientinum* L. were purchased from an authorized grain shop, Krishi Raksha Kendra, located in Bilaspur, India.

2.2. Study site and collection of samples

BMDE and degraded sludge samples were obtained from a large distillery located in Unnao having co-ordinates latitude 25.45° North and longitude 81.84° East. The effluent was collected in acid cleaned plastic bottles (5 L capacity) from the storage tank, nearby the oxidation ponds, where BMDE was kept in open environmental conditions for several months before its final disposal into the environment. The temperature, pH, and EC of effluent were measured on-site during its collection. The effluent had alkaline in nature (pH 8.1), dark-brown in appearance, with high EC (3.59 $\mu S\ cm^{-1}$). Apart from this, the degraded sludge was collected in plastic bags from the open land, located in premises of the industry, where sludge was dumped after biomethanation. The collected samples were immediately marked according to their source and location and delivered to the Bio-Resource Tech Laboratory in an ice-cold condition and kept at 4 °C until the performing the analyses, isolation of indigenous bacteria, and decolourization experiment.

2.3. Medium for decolourization study

The medium composition to start the decolourization experiment was selected from the previous reports of Chandra et al. (2018). The composition of medium in per liter of distilled water was glucose 1%, peptone 0.5%, $MgSO_4{\cdot}7H_2O$ 0.05%, and K_2HPO_4 0.1% (w/v). 1 M NaOH or 1 N HCL solution was used for the adjustment of medium pH 7.

2.4. Physico-chemical analyses of distillery effluent

BMDE (before and after biotreatment) was characterized to determine the content of various physico-chemical parameters, such as BOD_5, COD, TDS, EC, pH, color intensity, TS, sodium, chloride, sulfate, phosphate, TN, and TSS as per standard protocol (APHA-AWWA-WPCF, 2017). To determine the metallic load, BMDE was simultaneously processed using the nitric–perchloric acid mixture as described in APHA (Method 3030H). After digestion, the quantity of metallic ions in filtrates was directly determined by atomic absorption spectrophotometer (ZEEnit 700, Analytic Jena, Germany). The specific analyses of each physico-chemical parameter and the corresponding standardized processes are presented in Table S1.

2.5. Isolation, screening, and evaluation of ligninolytic activity of bacterial isolates

The potent melanoidin degrading bacteria were isolated from disposed sludge by selective enrichment approach as described by Kumar and Chandra (2018). Briefly, 10 g sludge sample was dissolved in Erlenmeyer flask containing 100 ml of sterile GPM broth consisting ($g\ L^{-1}$) glucose 1%, peptone 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, and K_2HPO_4 0.1% and flask then incubated in a rotary shaker at 120 rpm and 37 ± 1° C for one week. Afterwards, the samples collected from the flask showing decolorization were serially diluted. Thereafter, 0.1 mL sample from the respective dilution (10^{-5} and 10^{-6}) was collected and spread on pre-prepared GPM agar plates supplemented with increasing concentration of melanoidins (1000, 2000, 3000, and 4000 mg L^{-1}; w/v) and plates were kept at 37 ± 1 °C for 24 h. The degree of decolorization around the colony of potential bacteria was observed from the bottom of the culture plate. The clear zone diameter of more than 1 cm around the colony was considered as potent isolates for melanoidin degradation. The bacterial isolates, showing the luxuriant growth at a high concentration of melanoidins, were carefully picked and further purified by repeated streaking on agar medium plates. Simultaneously, the purified bacterial isolates having high melanoidins decolourization capability were selected and secondary screened to check their laccase activity (required for effluent biodegradation) on Boyd and Kohlmeyer (B&K) agar medium plates supplemented with 0.01% guaiacol by the standard plate assay method. B&K agar was contained 10 g glucose, 2 g peptone, 1 g yeast extract, and 20 g agar dissolved in 1 liter of distilled water. Pre-isolated bacteria were inoculated on B&K agar plates and were incubated at 37 ± 1 °C for one week. The bacterial isolates producing laccase was visually detected on guaiacol-agar plates by the appearance of brown colored zone around the

bacterial growth. Simultaneously, the isolates were also screened to detect the LiP activity on MSM agar plate containing (g L^{-1}) glucose 2.0; yeast extract 2.0; KCl 0.2; $MgSO_4 \cdot 7H_2O$ 0.2; $(NH_4)_2HPO_4$ 1.0; and agar powder 2.0 amended with azure-B solution (0.01%) as an indicator (Archibald, 1992). Afterwards, the plates were inculcated with pure bacterial of isolates and placed at 37 ± 1 °C for 48 h.

2.6. Bacterial identification

2.6.1. Physiological and morphological characterization

Isolated bacterial strain was characterized by using different biochemical tests, such as Gram and endospore staining, catalase, citrate utilization, hydrogen sulfide (H_2S) production, IMViC (I" is for indole test; "M" is for methyl red test; "V" is for Voges–Proskauer test, and "C" is for citrate test) as per the standard method described elsewhere (Holt et al., 1994).

2.6.2. Molecular identification

2.6.2.1. Genomic DNA isolation, 16s rDNA gene amplification and sequencing. The identification of potential bacterium was accomplished by gene sequence analysis of amplified region of 16S rDNA. For this, genomic DNA from screened bacterial strain was isolated by a standard protocol (Nnamchi et al., 2006). Afterwards, the 16S rDNA of isolate was amplified using reverse primer 492R (5′ − *TACGGCTACCTTGTTACGACTT* − 3) and forward primer F27 (5′ − *AGAGTTTGATCCTGGCTCAG* − 3) (dos Santos et al., 2019). Subsequently, DNA concentration was measured at 260/280 nm by® ND-1000 Nanodrop spectrophotometers. Next, electrophoresis was performed with 1% agarose gel to check the purity of DNA and its visualized using a Gel Documentation System. The resulting DNA from the gel was purified and sent for sequencing and bioinformatics analysis at the Biokart India Pvt Ltd., Bangalore, India. The gene sequencing was performed on ABI 3130 Genetic Analyzer. The generated reverse and forward sequences were assembled and a consensus sequence was generated.

2.6.2.2. Phylogenetic analysis. The 16S rDNA gene (partial) sequence of bacterium was compared against the neighbor consensus sequence existing in the GenBank database of NCBI using the BLASTN program available at NCBI server (Altschul et al., 1990). A total of nine16S rDNA gene sequences that showed maximum relatedness (95%–97%) with query sequences were imported from the GeneBank and these multiple sequences were aligned by ClustalW program to obtain a similarity score (Thompson et al., 2002). Afterwards, the multiple aligned sequence file was imported into the MEGA (version 11.0) to construct a phylogenetic tree (Tamura et al., 2021). To obtain the accession number, the generated consensus sequence of 16S rRNA gene of isolate was deposited to GenBank of NCBI.

2.7. Evaluation of effluent decolourization and biodegradation experiments

In order to assess the decolourization efficiency, the experiment was accomplished *in*-vitro using 250 ml Erlenmeyer flasks containing 10 ml of anaerobically treated spent wash supplemented with 90 mL of autoclaved decolourization medium. The medium composition was described earlier in Section 2.3. The medium pH 7.0 was adjusted and sterilized at standard conditions for 20 min. Afterwards, the tested flasks, inoculated with 1.0 ml of 24 h grown culture of isolate, were incubated at 37 ± 1 °C for 168 h in shaking (120 rpm) and static conditions. The samples (2 ml) at every 24 h interval was withdrawn from the inoculated flask and centrifuged at 10,000 ×g and 4 °C for 10 min to obtain the cell free supernatant. The supernatant fluid was then analyzed for reduction of color, BOD, COD etc. The decolorization experiments were performed in triplicate along with a similar set of abiotic controls (without bacterial inoculation). The effluent's color removal efficiency of the bacterial isolate was expressed in terms of percent decolorization monitored by a double beam UV–Vis spectrophotometer at respective λ_{max} (475 nm) of the supernatant obtained from the liquid culture medium upon centrifugation against un-inoculated effluent. The effluent decolourization efficiency was calculated as per Eq. (1):

$$D\ (\%) = \frac{(A_0) - (A_1)}{A_0} \times 100 \tag{1}$$

Where;

D = decolourization percentage

A_0 = the absorbance of distillery effluent at $t = 0$ min

A_1 = the absorbance of distillery effluent at time $t = t$ (min)

2.8. Influence of process parameters for efficient effluent decolorization

The effect of different carbon and nitrogen supplements along with abiotic factors was investigated to monitor the optimal conditions to be required for maximum decolorization of BMDE. Thus, the efficacy of effluent treatment by isolated bacterium strain VKDS9 was evaluated under various nutritional sources (carbon sources; w/v 1% mannitol, fructose, galactose, ribose, xylose, starch, glucose, and sucrose; nitrogen sources; 0.5% w/v ammonium nitrate, yeast extract, peptone, beef extract, ammonium chloride, urea, and sodium sulfate) and environmental process parameters like pH (5–9), temperature range (25–45 °C), agitation speed (150–200 rpm), and inoculum size (1–6 ml). A batch study with single process parameter optimization was carried out in 250 mL Erlenmeyer flasks containing 99 ml sterilized BMDE supplemented with the minimal media and inoculated with strain VKDS9 for up to 168 h in a shaking condition.

2.9. Qualitative determination of ligninolytic enzyme activity

Enzyme assays were conducted for laccase, MnP, and LiP at 24 h intervals till 168 h from the decolorization culture filtrates to measure the ligninolytic activities. The decolourized effluent was taken from a flask and then centrifuged at 10000 ×g for 10 min at 4 °C to obtain the crude enzyme extract. Afterwards, clear fluid was taken and then used for enzyme assay. Phenol red was used to measure the MnP activity as per standard procedure outlined by Wariishi et al. (1992). LiP activity was measured according to the method of Arora and Gill (2001) whereas laccase activity of supernatant was qualitatively determined by the standard protocol as outlined by Arora et al. (2002). This method is mainly based on guaiacol's oxidation. The optical density of supernatant was measured at 450 nm at every 1 min interval at 25 °C. The laccase and MnP enzyme activity are expressed as UL^{-1} of substrate oxidized per liter per minute and was calculated by Eq. (2) (Desai et al., 2011).

$$UL^{-1}min^{-1} = \Delta_{Abs} \times V/v \times \varepsilon \times T \quad (2)$$

Where; Δ_{Abs} = change in absorbance in per minute at 450 nm
ε = extinction coefficient of each substrate at particular wavelength (mol^{-1} cm^{-1})
V = final volume of the reaction mixture in milliliter
v = enzyme volume (ml)
T = reaction time in minute
One unit (U) of enzyme activity was defined as the activity of enzyme that catalyzed the oxidation of 1 M of substrate per min.

2.10. Determination of bacterial growth

Bacterial growth during effluent decolourization was monitored by double beam UV–Vis spectrophotometer. For this, the cell pellet obtained on centrifugation (10000 ×g at 4 °C for 15 min) was re-inoculated in double-deionized water (2 ml) and its absorbance was calculated at 620 nm. Simultaneously, CFU was determined at every 24 h interval till 168 h to check the growth of strain VKDS9 during effluent decolorization. SEM analysis was also done to investigate the effect of BMDE on the surface morphology of bacterial strain. For SEM analysis, 168 h old bacterial culture was harvested from the flask and then washed with 0.1 M phosphate buffer (pH 7.4). Thereafter, chemically fixed sample was examined under SEM. Simultaneously, the qualitative analysis of the elemental composition of bacterial biomass was analyzed by SEM coupled with EDX spectroscopy system (JEOL JSM-6490LV, US). SEM system was operated at 200 kV, counting time 60 s, and probe current 45 nA.

2.11. Evaluation of effluent's biodegradation and metabolites analysis

2.11.1. Solvent extraction and sample pre-processing

To extract a broad range of organic pollutants and metabolites from cell-free supernatant fractions of untreated and bacterial treated BMDE, liquid–liquid extraction was subjected with ethyl acetate as per the method of Kumar et al. (2021). Before the extraction procedure, samples (untreated and treated effluent) were centrifuged at 10000 ×g 4 °C min for 15 min followed by filtration through a glass microfiber filter (47 mm; Whatman™) to eliminate the undissolved solid particles. The extraction process was repeated thrice with fresh solvent for utmost extraction of metabolites. All the extracts obtained from the organic layer were filtered through 0.22-μm syringe filter, dewatered with anhydrous sodium sulfate, and then evaporated using a vacuum rotator evaporator at 25 °C till dryness. Next, dry residues were dissolved in 1 ml methanol and then passed through 0.22-μm syringe filter. The filtrate was used for further analyses.

2.11.2. HPLC analysis

The initial and bacterial treated sample of BMDE was also analyzed by 515 HPLC system coupled with a diode array detector system (1100 series, Agilent Technologies, USA) and C18 reverse phase column (250 × 4.6 mm, 5 μm particle size) using the gradient of solvent A (acetonitrile with 0.1% TFA) and Mili-Q water in ratio of 70:30 (v/v) as mobile phase at a flow rate of 0.4 mL min^{-1} (Chandra et al., 2018; Yadav and Chandra, 2012). The injected volume of sample was 50 μl. In order to assess the degradation of melanoidins present in BMDE, the detection was observed at wavelength of 250 nm.

2.11.3. Mas spectroscopy analysis

GC–MS analysis was performed to detect and characterize the metabolites of BMDE generated after bacterial biodegradation. Prior to GC–MS analysis, the samples were derivatized to TMS as described earlier (Chandra and Kumar, 2017b). Subsequently, the metabolites produced during biodegradation and decolourization of BMDE were identified in concentrated extracts by Gas Chromatograph coupled to a single quadrupole mass spectrometer (QP2010 SE, Shimadzu). The separation of metabolites was achieved through DB-5MS capillary column. The operational conditions for the analyses were defaulted according to Kumar and Chandra (2020). EI mass spectrum of metabolites eluted from the GC column was identified by matching EI mass spectra to standard NIST mass spectra library database inbuild with GC–MS.

2.12. Phytotoxicity assessment

The phytotoxicity effect of effluent, before and after bacterial treatment, was investigated to determine their impact on the plants once it is disposed into the environment. Thus, the test was performed using the uniform seeds of *C. arientinum* L. To accomplish the germination assay, surface-sterilized seeds (ten seeds) were kept on a double layer of UV-sterilized filter paper in each Petri dish irrigated with 8 ml of different dilutions of BMDE (10, 20, 30, 40, and 50 v/v), before and after treatment with bacteria. Subsequently, all Petri plates were kept in an incubator at 25 ± 1° C in dark condition for germination. Seeds, where over 2 mm long, were considered to be germinated. At the end of 5 days of the experiment, the root and shoot elongation in germinating seed was measured. Control assays were carried out using 8 ml of deionized water at the same time. To determine the degree of inhibition over control, the toxicity of BMDE was calculated as per Eq. (3) through 12 (Salem et al., 2015; Wani et al., 2019; Jagadabhi et al., 2019; Rashid et al., 2021)

$$\text{Relative Seed germination, RSG (\%)} = S_t/S_c \times 100 \quad (3)$$

$$\text{Germination index, GI} = N_T \times L_T\%/N_C \times L_C \quad (4)$$

$$\text{Inhibition percentage} = A_c - B_t/A_c \times 100 \quad (5)$$

$$\text{RRG (\%)} = MR_T/MR_C \times 100 \quad (6)$$

$$\text{Phytotoxicity index, PI} = 1 - RL_T/RL_C \quad (7)$$

$$\text{Phytotoxicity (\%)} = RL_C - RL_T/RL_C \times 100 \quad (8)$$

$$\text{Seedling vigor index (SVI)} = \text{Germination percentage} \times \text{seedling length} \quad (9)$$

$$\text{Tolerance index} = \text{Root length in treatment/Root length in control} \quad (10)$$

$$\text{Root Shoot ratio} = \text{Average root length/Average shoot length} \quad (11)$$

$$\text{Seedling mortality rate} = \text{Total seeds} - \text{total number of germinated seeds} \quad (12)$$

Where;

St = total number of germinating seeds in treated effluent; Sc is the number of seeds in untreated effluent; SG%=seed germination percentage; N_T = total number of seeds germinated in tested effluent; L_T; average length of root in tested effluent; N_C = Total number of seeds germinated in untreated effluent; L_C = total number of seeds germinated in untreated effluent; RE% = relative root elongation percentage; I = inhibition percentage; A = seed germination percentage in control sample; B=seed germination percentage in tested sample; RRG = relative root growth; MR_C = mean root length in control sample; MR_T = mean root length in tested sample; RL_C = radical length in untreated effluent; RL_T = radical length in treated effluent; RL_T = root length of tested seeds; RL_C = root length in untreated effluent

2.13. Data analysis

All the experiments were performed in three replicates. Results of triplicates were combined and represented as mean ± standard deviation (SD) calculated by standard application Excel Spreadsheet (Microsoft Office 2019). Combined mean values were subsequently subjected to ANOVA by SPSS (v. 19.0 SPSS, USA) and the least significant difference was carried out.

3. Results and discussion

3.1. Isolation, screening, and melanoidin degrading activity of ligninolytic bacterial isolate

Microorganisms flourishing on toxic and refractory chemicals containing distillery sludge disposed area probably make up the potential to adapt themselves over the ages to degrade these compounds. The sludge microenvironment is an effective incubator of a wide variety of potent bacteria and offers an ideal source for the isolation of potent indigenous bacteria (Chandra and Kumar, 2017a,b). In the current study, total nine aerobic bacterial isolates were cultured from distillery sludge. Out of nine strains, one bacterial strain VKDS9 showed higher melanoidin degrading activity on agar medium plate methods (Table S2). Melanoidin is the major recalcitrant polymer formed by amino-carbonyl reactions which is discharged in biomethanated distillery wastewater. The characteristic dark brown color of distillery effluent even after anaerobic treatment is mainly attributed due to the presence of melanoidins. The isolated strain was also capable of producing laccase, MnP, and LiP as prominent ligninolytic enzymes in vitro plate assay method (Table S2). The guaiacol, a phenolic naturally occurring organic compounds, was widely used for quick detection of laccases producing microbial strains by means of a microbial catalyzed oxidation of phenolic substrate to form reddish-brown halo zones in the medium. Bacterial strain produced a yellow zone after the first day of incubation, indicating the MnP activity while the disappearance of the blue color of Azure-B indicated the peroxidase enzyme activity in vitro plate assay. The observation of this study was similar with the earlier results for screening of ligninolytic enzymes producing microbes which have been reported by various workers (Chandra and Singh, 2012; Falade et al., 2017; Gaur et al., 2018).

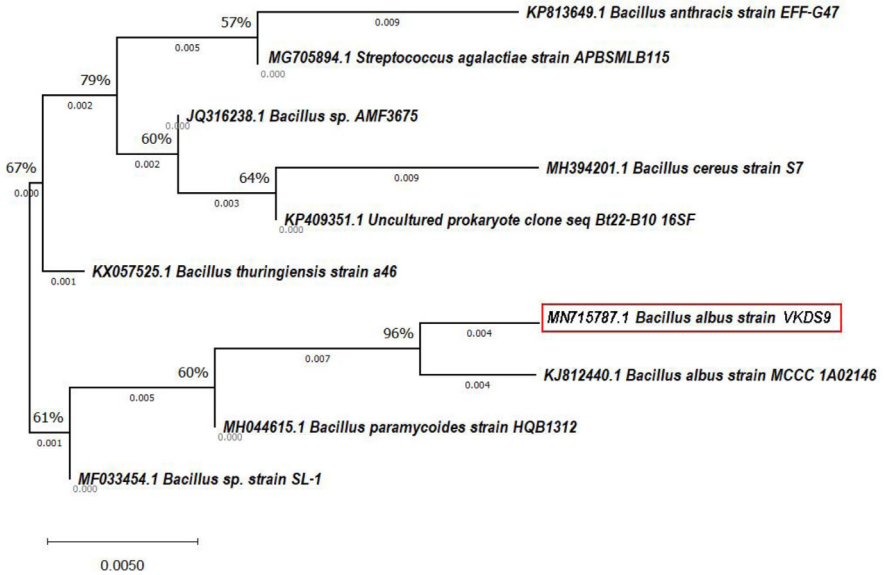

Fig. 1. Phylogenetic tree and evolutionary analyses was inferred by using the Neighbor-Joining method with 1000 bootstrap values in MEGA 11.0. This tree showing interrelationship of isolated strains with most closely related species inferred from 16S rRNA sequences. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The values on the nodes indicate the similarity percentage among the bacterial strains.

3.2. Identification of bacterial isolate VKDS9

The results of conventional test for biochemical characterization showed the bacterial isolate VKDS9 morphologically was rod-shaped, gram-positive, motile, and endospore former. It is biochemically, catalase, starch hydrolysis, oxidase, and gelatinase positive with fermentative nature and positive for the glucose and maltose and negative for arabinose and arabitol. The strain was also negative for urease, indole, hydrogen sulfide, methyl red and citrate utilization. It shows luxuriant growth between pH 5.0 and 8.0. Based on morphology, physiology, and biochemical tests, the strain VKDS9 was identified as *Bacillus.* To confirm the identification at a species level, bacteria strain VKDS9 was also identified by 16S rDNA gene sequencing analysis. Results of BLASTN search showed highest sequence homology (96%) of bacterial isolate VKDS9 with *Bacillus albus* strain MCCC (Accession No. KJ812440) and can be considered a novel strain. The generated phylogram showed the closest homology of VKDS9 with *B. albus.* Fig. 1 shows the phylogenetic relationship of 16S rRNA gene sequence (1442 bp partial sequence) of VKDS9 and other species and strains correlated to *B. albus* in GenBank. Thus, on the basis of sequence similarity index and phylogenetic analysis of 16S rDNA sequence, the isolate VKDS9 was identified as a new strain of *B. albus*. This bacterium is classified into phylum, Firmicutes. The allocated GenBank accession number of *B. albus* strain VKDS9 is MN715787.

3.3. Physico-chemical characterization of effluent before and after bacterial treatment

The average color range, temperature, and EC of dark-brown BMDE was found to be 21257 Pt–Co units, 34 ° C, and 3.59 mS cm^{-1}, respectively, which was higher than the recommended set values of effluent discharged as prescribed by CPCB (2016). The intense dark-brown color of BMDE is due to the presence of melanoidins, caramel, alkaline degradation products and polyphenols, etc. The pH of untreated BMDE was mainly alkaline (pH 8.05) in nature. The extreme color of BMDE was 21257 co-pt, which hindering the sunlight penetration and ultimately inhibited the photosynthetic reaction of aquatic organisms. BOD_5, COD, TSS, and TDS values were measured as 7457.5 mg/l, 12294 mg/l, 2457 mg/l, and 362.5 mg/l, respectively, which indicates that the effluent was highly polluted severely posing depletion of natural resources and also posed a risk to the aquatic organisms and human health. BOD is considered to be a principal indicator of organic pollution which indicates the existence of easily biodegradable organic matter in water/wastewater. In the current study, the detected values of COD were elevated than BOD as presented in Table 1. The high COD might be attributed to high concentration of biologically resistant substances, which remain unaffected by anaerobic treatment. Therefore, distillery effluent must be adequately remediated before its final disposal into the ecosystem. The physico-chemical analysis of the untreated effluent had shown a high concentration of chloride (347 mg/l), sulfate (164 mg/l), phosphate (526 mg/l), sodium (136.5 mg/l), ammonical nitrogen (461.5 mg/l) and color(Table 1) that contributed alkalinity to the effluent. The effluent was found to a had high content of metallic ions, which was higher than the recommended permissible limits set by CPCB (2016). The analysis results illustrate that all the values of physicochemical parameters of untreated effluent

Table 1
General physicochemical characteristics of biomethanated distillery effluent before and after bacterial treatment.

Parameter	Unit	Condition	Min.	Max.	Mean	SD	Permissible level*
Color appearance	–	Untreated	–				NS
	–	Treated	–				NS
Color Intensity	Co-Pt	Untreated	21256	21259	21257	32.00	NS
	Co-Pt	Treated	4226	4237	4236.5[a]	8.25	NS
pH	–	Untreated	8.00	8.10	8.05	0.11	5.5–9.0
	–	Treated	7.5	7.1	7.3[c]	0.02	5.5–9.0
EC	$\mu S\ cm^{-1}$	Untreated	3.57	3.61	3.59	0.02	0.4
	$\mu S\ cm^{-1}$	Treated	2.15	2.21	2.18[c]	0.01	0.4
Temperature	^0C	Untreated	31	34	32.5	1.05	NS
	^0C	Treated	37	37	37[ns]	0.00	NS
BOD	$mg\ L^{-1}$	Untreated	7456	7459	7457.5	25.14	30.00
	$mg\ L^{-1}$	Treated	3259	3293	3261[a]	5.00	30.00
COD	$mg\ L^{-1}$	Untreated	12292	12296	12294	14.00	250.00
	$mg\ L^{-1}$	Treated	7869	7872	7870.5[a]	11.26	250.00
TS	$mg\ L^{-1}$	Untreated	241	243	242	2.00	NS
	$mg\ L^{-1}$	Treated	152	153	152.5[a]	1.36	NS
Sodium	$mg\ L^{-1}$	Untreated	135	138	136.5	1.26	NS
	$mg\ L^{-1}$	Treated	69	70	69.5[a]	0.26	NS
Chloride	$mg\ L^{-1}$	Untreated	345	349	347	3.59	600
	$mg\ L^{-1}$	Treated	135	139	137[a]	1.02	600
Sulfate	$mg\ L^{-1}$	Untreated	165	163	164	1.74	NS
	$mg\ L^{-1}$	Treated	75	76	75.5[a]	0.55	NS
Phosphate	$mg\ L^{-1}$	Untreated	524	528	526	5.00	NS
	$mg\ L^{-1}$	Treated	216	219	217.5[a]	2.65	NS
TN	$mg\ L^{-1}$	Untreated	685	691	688	6.00	NS
	$mg\ L^{-1}$	Treated	426	427	426.5[a]	5.36	NS
TSS	$mg\ L^{-1}$	Untreated	362	363	362.5	4.26	100.00
	$mg\ L^{-1}$	Treated	96	98	97[a]	0.22	100.00
TDS	$mg\ L^{-1}$	Untreated	2456	2458	2457	9.00	2100.00
	$mg\ L^{-1}$	Treated	426	430	428[a]	4.25	2100.00
NH_4^+-N	$mg\ L^{-1}$	Untreated	461	462	461.5	3.56	NS
	$mg\ L^{-1}$	Treated	45	46	45.5[a]	0.02	NS
Iron (Fe)	$mg\ L^{-1}$	Untreated	23.12	23.15	23.13	0.15	NS
	$mg\ L^{-1}$	Treated	15.22	15.23	15.22[a]	0.29	NS
Zinc (Zn)	$mg\ L^{-1}$	Untreated	0.231	0.234	0.232	0.08	NS
	$mg\ L^{-1}$	Treated	0.042	0.045	0.043[a]	0.00	NS
Manganese (Mn)	$mg\ L^{-1}$	Untreated	1.264	1.267	1.265	0.09	NS
	$mg\ L^{-1}$	Treated	0.241	0.242	0.241[a]	0.02	NS
Copper (Cu)	$mg\ L^{-1}$	Untreated	0.141	0.144	0.142	0.04	NS
	$mg\ L^{-1}$	Treated	BDL	BDL	BDL	–	NS
Nickel (Ni)	$mg\ L^{-1}$	Untreated	0.321	0.322	0.321	0.02	NS
	$mg\ L^{-1}$	Treated	0.025	0.026	0.025[a]	0.00	NS
Chromium (Cr)	$mg\ L^{-1}$	Untreated	0.061	0.064	0.062	0.01	NS
	$mg\ L^{-1}$	Treated	0.021	0.026	0.023[b]	0.00	NS

SD: Standard deviation; NS: Not specified; EC: Electrical conductivity; BOD: Biological oxygen demand; COD: Chemical oxygen demand; TS: Total solids, TN: Total nitrogen; TSS: Total suspended solids; TDS: Total dissolved solids; BDL: Below detection limit; * A general and specific standard criteria for the discharge of effluent into inland surface waters recommended by MoEF&CC (CPCB, 2016). All the values are mean ±SD (n = 3); Students *t* test (two tailed as compared to untreated biomethanated distillery effluent); [c]Less significant at $p < 0.05$; [ns]Non significant at $p > 0.05$; [a]Highly significant at $p < 0.001$; [b]Significant at $p < 0.01$.

as presented in Table 1 were found beyond the discharge limits prescribed by CPCB which indicated that it has the potential to pollute the environment. Thus, the detoxification and decolorization of BMDE was further performed using novel bacterial isolate, *B. albus* strain VKDS9. In the present study, heavy metals discharged within distillery waste were found to be extremely higher than the permissible limits of CPCB (2016) (Table 1) which pose a critical risk to human and the environmental health.

In contrast to untreated effluent, the color of the effluent (1760 Pt–Co units) was reduced to 4236.5 Pt–Co units after bacterial remediation. Initially, during degradation of BMDE, the pH of the medium was decreased slightly to 7.3. Moreover, there was a significant reduction in other physicochemical parameters after bacterial treatment with 144 h of incubation period. The results also showed that BOD, COD, and TDS values along with color were decreased sharply after bacterial treatment at 144 h of incubation as presented in Table 1. In addition, maximum removal of metals was also observed by growing bacterial cells due to metabolization. Significant reduction in BOD, COD, and TDS along with color and metals values of BMDE by microbial isolates has also been reported by several workers (Mohana et al., 2007; Chowdhary et al., 2020). However, a significant removal of pollution load in BMDE after bacterial treatment was observed. The resulted BMDE still retain high value of various pollution parameters including BOD and COD which was higher than

the limits of effluent discharge as recommended by various regulatory agencies. Thus, further research is urgently required to optimize the system to achieve the maximize pollution remediation by the bacterial strains. Table 1 summarizes the physicochemical characteristics of untreated and bacterial treated BMDE during the validation studies. Significant factor's effects ($p < 0.05$) are also indicated in Table 1.

3.4. Process parameters optimization

An imperative job for the remediation of wastewater is the discovery of efficient microorganism(s) together with the findings of optimal culture conditions by adding different additional carbon and nitrogen co-substrates in initiating microbial growth and related decolorization to deal with the pollutants present in specific environments. In order to maximize the decolourization, different carbon and nitrogen supplements were scrutinized to optimize the BMDE treatment process by bacterial culture within short period. In the present study, glucose, a six-carbon sugar generally required for growth, was found as an efficient carbon source that resulted the decolourization of BMDE up to 79% along with reduction of BOD, COD, and TDS and other physico-chemical parameters after 144 h of incubation. However, mannitol, fructose, galactose, ribose, xylose, starch, and fructose were showed decolourization of BMDE up to 35%–70% as compared to glucose (Fig. 2a). No decolourization was reported in the control set incubated without microbial inoculation. Several studies have stated the effect of various carbon sources on the melanoidin degradation in connection of decolourization of distillery effluent (Tiwari et al., 2012; Santal et al., 2016). The major reason behind the maximum decolourization of BMDE in the presence of glucose is that the addition of different carbon co-substrates in media act as electron donor and induce the synthesis of ligninolytic enzymes which initiate the decolourization of BMDE. Apart from carbon sources, the effect of different nitrogen sources in culture medium for the decolourization of BMDE by bacterial strains was studied. Among different nitrogen sources, peptone, a simple nitrogen source necessary for microbial growth, improved decolourization of BMDE efficiently up to 55% in static conditions. However, the presence of other organic nitrogen sources, such as ammonium nitrate, beef extract, yeast extract, ammonium chloride, urea, and sodium sulfate could show the effluent decolourization between 25% and 75% only (Fig. 2b). Similar to the results of the present study, Tiwari et al. (2012) reported that nitrogen is the best supplement which efficiently decolourized distillery effluent under static conditions. The reduction in COD, BOD along with color reported earlier by various researchers through axenic or mixed bacterial culture utilized glucose and peptone as best carbon and nitrogen sources for their growth, respectively (Mohana et al., 2007; Tiwari et al., 2014; Kumar and Chandra, 2018). Fig. 2c–d shows the individual effects of temperature, pH, and agitation sped on decolourization of BMDE.

Simultaneously, the effects of various abiotic factors i.e. agitation, temperature, and pH on the decolorization and degradation performance of BMDE by *Bacillus albus* strain VKDS9 were studied in detail. Thus, the effect of pH along with shaking speeds on decolourization efficiency of strain VKDS9 was studied in the range of 5–9. In this study, the maximum decolorization up to 81% was achieved at pH of 8.0 with agitation speed of 180 rpm after incubation of 144 h (Fig. 2c). Further, changes in pH and shaking speed decrease in degradation and decolorization rate of BMDE. pH is one of the major parameters that influence the rate of decolourization. An increase or decrease pH thereby inhibited the enzymatic activity of bacterial strain. Effluent degradation in connection of decolorization is an oxidative process; thus, aeration work as a function of oxygen concentration speeds up the decolorization process. In the present study, it was observed that an increase in shaking speed resulted in a reduction in decolourization.

This might be due to the mechanical injury of bacterium cells at higher shaking speeds. Several studies have reported the optimal decolorization and degradation of melanoidin containing wastewater in shaking flask conditions ((Jiranuntipon et al., 2009; Chandra et al., 2018). Apart from pH and agitation, temperature also plays a key role in many decolourization processes that regulates microbial activity. The effect of pH on BMDE was studied as a function of temperature at different time intervals as depicted in Fig. 2d. It was observed that decolourization increased with an increase in temperature from 25 to 37 °C. In this study, utmost 83% decolourization efficiency was observed at 37 °C. Further, an increase in temperature resulted in slower growth of microorganisms and less removal of chemical pollutants. Previous studies showed the reduction in color and simultaneous removal of melanoidins (Tiwari et al., 2013). The microbial growth response and effluent degradation in connection to decolorization increased with inoculum size and recorded up to 168-h incubation. In the present study, when the inoculum size was increased up to 2.0% (v/v), the bacterial growth and BMDE decolorization was increased in synchrony with each other. It was observed that decolorization percentage enhanced with an increase in inoculum size of *B. albus* strain VKDS9 from 1 to 3%. However, further increase in inoculum size to 3, 4 and 5% (v/v) resulted no changes in BMDE decolorization. The optimum effluent decolorization (84%) was observed at 2.0% (v/v) inoculum of *B. albus* inoculum within 144 h. Thus, 2.0% (v/v) dose of bacterial inoculum was selected as optimum value for further effluent decolorization studies.

3.5. Ligninolytic activity

A major mechanism behind microbial biodegradation and decolourization of industrial effluent is because of the activity of a network of versatile biotransformation enzymes. Several microbes including bacteria possess a highly potential

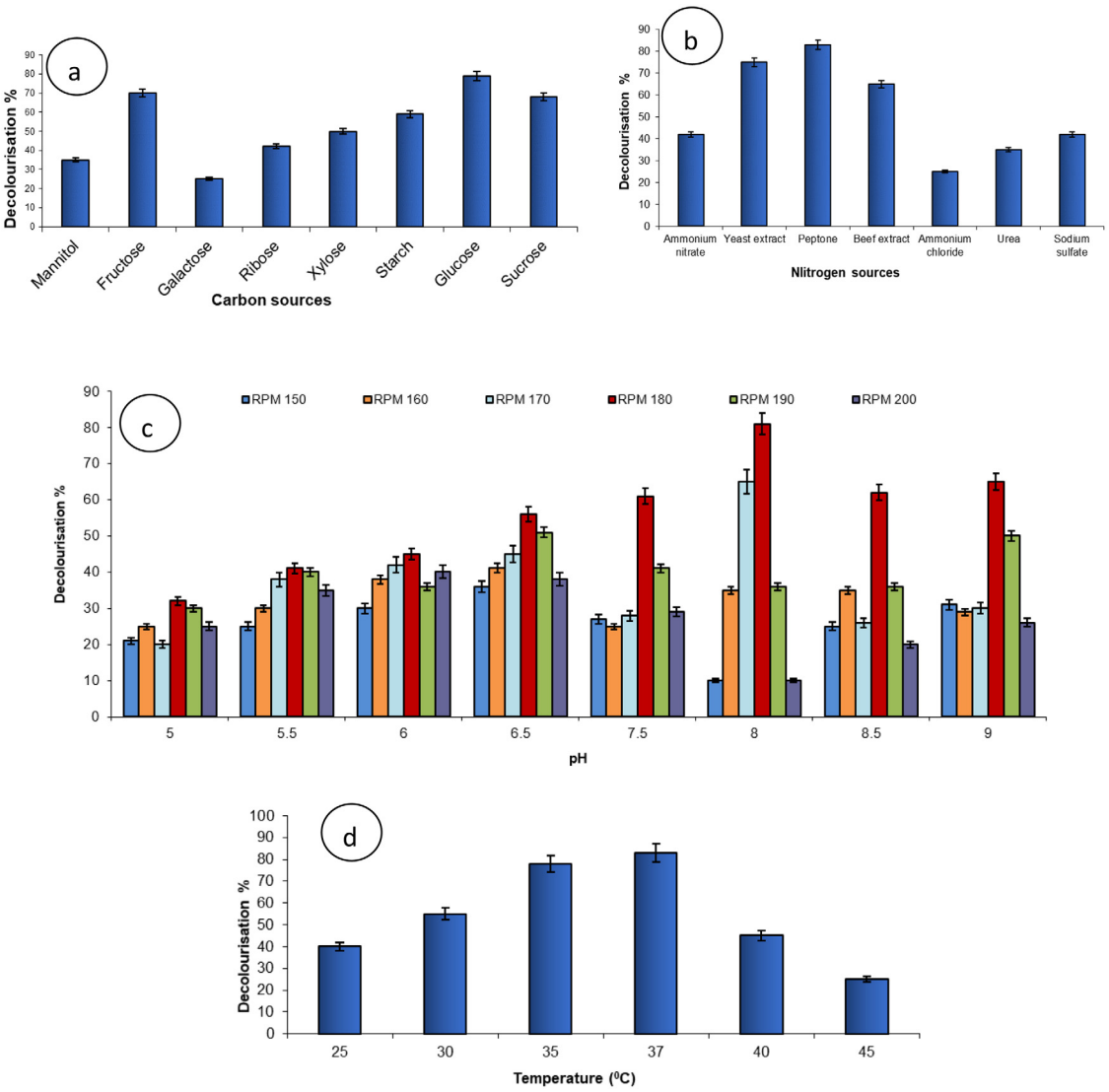

Fig. 2. Effect of various nutritional and abiotic process parameters on distillery effluent decolourization (a) carbon sources (b) nitrogen sources, (c) pH and agitation speed (d) and temperature at 37 °C up to 144 h incubation.

ligninolytic system which efficiently decolorize coloring molecules as well as various refractory organic pollutants. In order to explore the insight of BMDE bioremediation by the *B. albus* strain VKDS9, the activities of extracellular ligninolytic enzymes were measured during decolourization and degradation in static conditions at different time intervals. The activity of MnP, and LiP was very low at 0 days of incubation while the activity of laccase was only 0.29 U L^{-1} (Fig. 3). However, extracellular activity laccase, MnP, and LiP significantly increased after 24 h by bacterial strain. During decolorization of BMDE by *B. albus* strain VKDS9, LiP activity was very low, while laccase and MnP were more active. The above observations demonstrated that BMDE was decolorized by the involvement of extracellular ligninolytic activities bacterial strain. However, extracellular laccase and MnP activity were significantly decreased after 144 h of incubation as compared to 0 h. However, laccase and MnP are the major substrates oxidizing enzymes that played a vital role in the remediation of wastewater pollutants due to attacking on a wide range of conjugated chemical bonds present in phenolic and non-phenolic recalcitrant chemicals. The maximum amount of laccase and MnP was recorded in the supernatant which confirms the predominant roles of these enzymes in degradation of melanoidin in connection to effluent decolourization. The existence of melanoidins, phenolics and other heterocyclic compounds can induce the expression of MnP and laccase genes, both described as having synergistically action in the depolymerization and degradation of melanoidins containing distillery effluent. The involvement of microbial laccase and MnP in decolorization of distillery effluent was documented earlier by González et al. (2008) and Miyata et al. (2000).

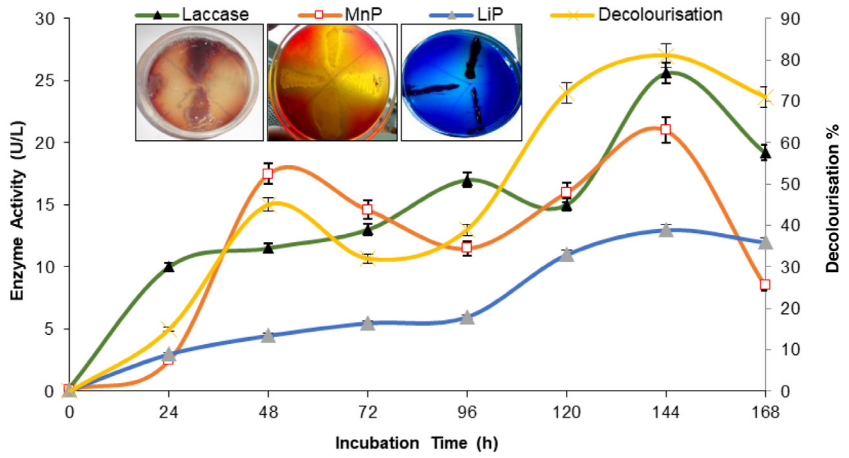

Fig. 3. Ligninolytic activity of *Bacillus albus* strain VKDS9 during decolourization and degradation of biomethanated distillery effluent under optimized nutritional and environmental conditions at different time interval.

3.6. Bacterial growth, biomass, and SEM-EDX

The periodic monitoring of bacterial growth was recorded in terms of increase in culture absorbance at 620 nm with a spectrophotometer. The bacterial biomass, on the other hand, was measured gravimetrically. It was observed that highest OD_{620}(2.79) and biomass (4.61 g L^{-1}; CFU/mL 25 $\times$ 10^5) were attained within 144 h incubation period during decolourization experiment (Fig. 4a,b). which led to less enzyme production. This indicated the optimal condition for the highest decolourization of BMDE at 144 h. The SEM of bacterial biomass was carried out to evaluate the changes in surface morphology of the bacterial cells after BMDE treatment at 144 h. It was noticed that the bacterial biomass showed thickness and bacterial cell surface was appeared rough with decreased stiffness (Fig. 4c). The results of the current study are well supported by earlier reports (Tiwari et al., 2012). Further, the SEM–EDS analysis of bacterial biomass showed heterogeneous and complex elemental composition (Fig. 4d). The EDS spectra revealed the presence of various major elements C (51.43%), O (20.94), F (13.07), along with insignificant traces of Mg (0.65%), P (0.01%), Ca (3.11), Zn (0.11), Ir (0.95) and Pt (9.72%) in the biomass at various energy levels. The present results are in accordance with the previously reported work of Tiwari et al. (2012).

3.7. Biodegradation analyses and metabolites characterization

Metabolism of BMDE is mainly due to enzyme machinery present in microorganisms. This was carried out using sophisticated HPLC and GC–MS techniques to reveal the probable mechanism of decolourization and degradation of BMDE and analyzed the generated metabolites before and after bacterial treatment

3.7.1. HPLC analysis

The HPLC elution profile of untreated BMDE showed the presence of several major peaks at RT of 2.783, 3.000, 3.542, 3.883, 4.075, 6.225, and few minor peaks at RT of 8.242, 9.542, and 10.425 min (Fig. 5a). These peaks were reduced after bacterial treatment at 144 h. In addition, the disappearance of the broad peaks was observed in the bacterial treated BMDE at a RT of 7.204 min. This resulted in the formation of new relative peaks at RT of 2.767, 2.975, 3.533, 6.233, 8.233, and 10.458 min (Fig. 5b). The formation of six minor peaks in decolorized metabolites of BMDE was also observed which was not seen in the untreated BMDE. This significant HPLC spectra difference between the untreated BMDE and its decolorized metabolites supports the biodegradation of BMDE into various metabolites.

3.7.2. GC–MS

GC coupled with mass spectrometer is an ideal sophisticated analytical technique and has been applied to determine the detect and characterize organic pollutants discharged in the environment from various industrial activities. In order to reveal the nature of metabolites generated by *B. albus* strain VKDS9, GC–MS analysis was performed on ethyl acetate extract compounds before and after biodegradation to investigate the metabolites. The metabolites detected by GC–MS in the organic extract of control and treated samples have been identified as trimethylsilyl (TMS) derivatives at the particular retention time compared with the mass spectra of the authentic compounds documented in the NIST library. The best matches are reported in Table 2. From Fig. S1a, the major peaks detected in the control samples at different RTs were pentadecane (RT 11.88), pyrazine, 2,5-dimethyl-3-propyl (RT 21.55), hexadecanoic acid, ethyl ester (26.55), dotriacontane (27.47), òctadecanoic acid, TMS (30.36), Methyl 19-methyl-eicosanoate (32.15), and octadecanoic acid,

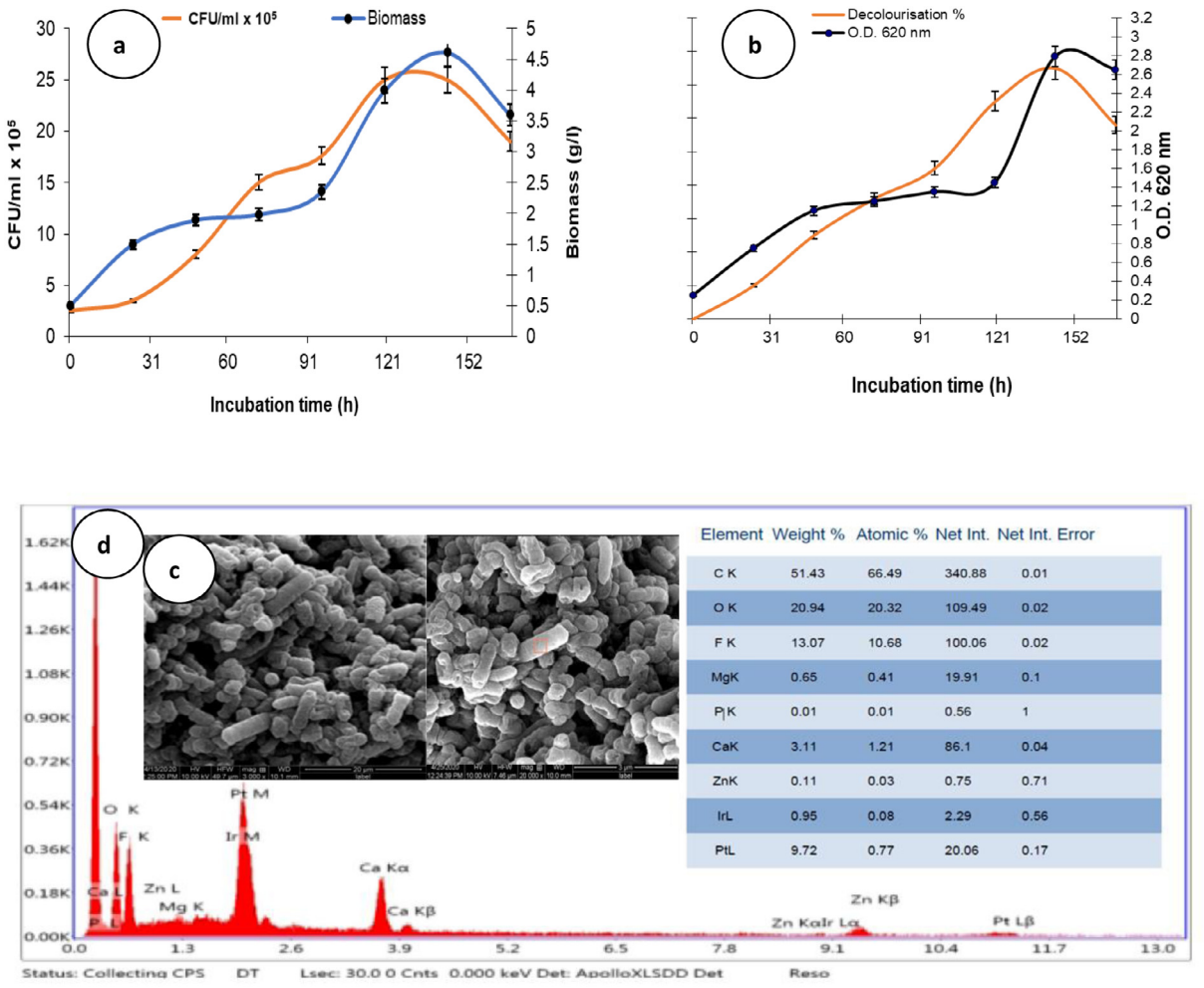

Fig. 4. Growth and elemental analysis (a-b) removal of color and growth profile of *Bacillus albus* strain VKDS9 during the decolorization of biomethanated distillery effluent (c–d) SEM–EDS analysis of bacterial mass during distillery effluent treatment under static conditions.

2,3-bis[(TMS)oxy]propyl ester (35.57). The major organic constituents that were present in high quantity in the fractions were identified. However, small peaks detected by GC–MS could not be identified. Some of the compounds detected in the untreated BMDE pose a serious risk to organisms often continuous input of pollutants/toxicants into aquatic ecosystems with long-term implications on ecosystem functioning. These pollutants remain in the environment and do not easily degrade during the anaerobic treatment and are discharged directly into the aquatic environment. The environmental impacts of the detected substances derived from in this study have also been reported elsewhere (United States Environmental Protection Agency (USEPA), 2012; Kumar and Chandra, 2020).

In contrast to the above, GC–MS chromatogram of the metabolites extracted from bacterial treated sample in repeated batch process after 144 h of treatment (Fig. S2b) revealed an enormous increase in the peaks' number at different RT and reduction of peaks as compared to the untreated sample, which indicated the generation of a large number of new metabolites from the BMDE as a consequence of bacterial degradation (Fig. S.2b). The major detected peaks at RT of 11.88, RT 21.55, 26.55, 27.47, 30.36, 32.15, and 35.57 min were disappeared after 144 h of incubation and some new major peaks at RT of 9.11 (ethanedioic acid, bis(TMS)ester), 13.72 (butenedioic acid, bis (TMS)ester), 34.50 (cleidoniol, deoxy), and 36.11 (2,6,10,14,18,22-Tetracohexaene,2,6,10,15,19,23-hexamethyl) emerged, specifying breakdown or mineralization of low-molecular-weight compounds as listed in Table 2. In addition, several minor peaks were observed at different RT, which corresponded to various metabolites. These new metabolites explore the degradation of the mechanism of effluent by bacterial culture. Since pure culture of the strain VKDS9 was used in the batch experiment with BMDE where organic pollutants were co-metabolically utilized in the presence of carbon and nitrogen sources and hence the gradual utilization of compounds by the bacteria possibly resulted in the reduction of a number of peaks during the treatment process. Degradation and transformation of organic and organometallic pollutants and metabolites formation

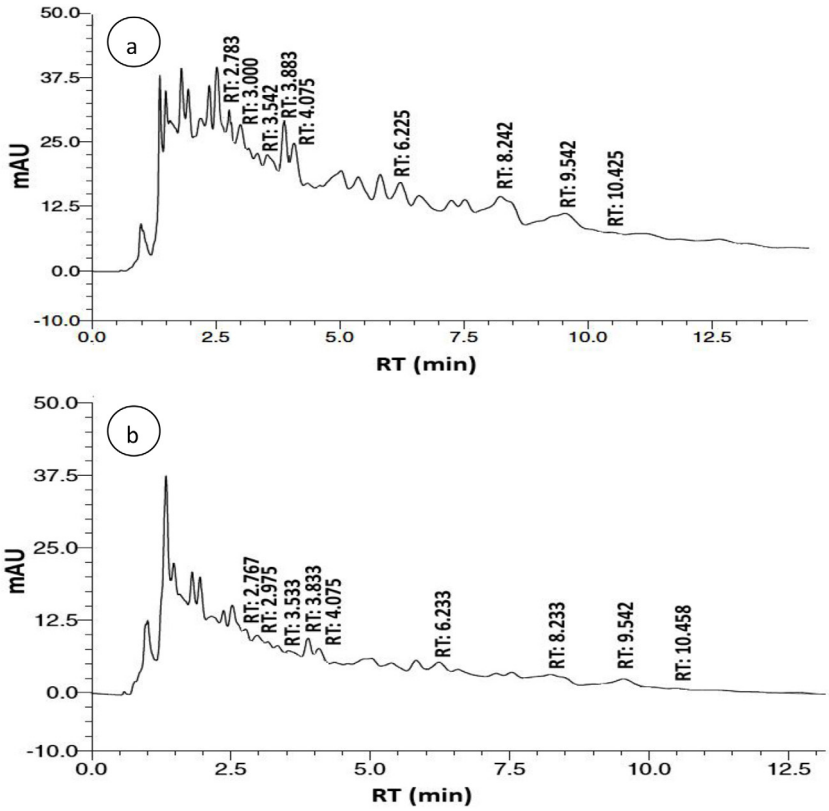

Fig. 5. HPLC chromatogram of (a) untreated biomethanated distillery effluent, and (b) its decolorization metabolites obtained after bacterial treatment at 144 h incubation under static conditions.

after microbial treatment of distillery effluent were also reported by Tripathi et al. (2021). These results indicated that color removal by strains VKDS9 might be largely attributed to biodegradation.

3.8. Toxicity assessment of distillery effluent for its safe disposal

In India, the improper disposal of untreated or partly treated distillery effluents, containing refractory pollutants, causes a serious impact on agricultural crops and soil microflora as this effluent is being used for fertiirigation (Kumar and Chopra, 2012; Chandraju et al., 2013). Seed germination and plant growth bioassays are relatively simple and precise toxicological tools are widely used to assess the phytotoxicity of chemical contaminants discharged in wastewater (Kodituwakku and Yatawara, 2020; Agrawal et al., 2021). Thus, the phytotoxic effects towards the distillery effluent and its metabolic products by bacterial isolate concerning *C. arientinum* L. was studied. The phytotoxicity study of untreated effluent showed only 100, 90, 80, and 60 percent germination at 10, 20, 30, and 40% different concentrations of BMDE, respectively as depicted in Fig. 6 and Table 3. In addition, 50% BMDE resulted in no germination and no plant growth. The root lengths and shoot lengths were decreased significantly when seeds were treated with different concentrations of untreated BMDE as compared with that of the water treated seeds as control. The untreated tested BMDE also showed 29.09, 36.36, 72.72, and 81.81 phytotoxicity percentage on seed. These results indicated that BMDE is markedly phytotoxic at certain concentrations. Fig. 6 shows that BMDE significantly affected the root and shoot elongation *C. arientinum* L. The exposure of the untreated BMDE adversely affect the root and shoot lengths of germinating seeds. It was observed that untreated BMDE at 30 and 40% completely retarded shoot germination of *C. arientinum* L. seeds. It has also been reported that the reduction of seedling growth parameters and seed germination might be attributed to the high toxic organic chemical, organometallic load, and high salt content that act as an inhibitor of different biochemical and physiological processes, especially alter the functioning of cytokinins, gibberellins, auxins, and amylases (Kumar and Chopra, 2012; Leiva et al., 2019; Šourková et al., 2020). In the present study, the effect of BMDE on various seedling growth parameters with increasing concentrations of BMDE was studied as presented in Table 3. The toxic effects of anaerobically digested spent wash on various growth parameters in germinating seeds were previously reported by Bharagava and Chandra (2010). Similarly, Ramana et al. (2002) demonstrated that germination of seed and seedling growth was continued to decline over the entire stress period. These results clearly indicated that discharged BMDE did impediment of seed germination and extremely toxic to vegetation and it must be adequately treated before its final dumping into the terrestrial environment.

Table 2
Compounds identified as trimethylsilyl (TMS) derivatives by GC–MS technique in ethyl acetate extract of biomethanated distillery effluent.

SI. No.	RT	Identified compounds	Area (%)	UTDE	BTDE
1.	7.35	Acetic acid, [(TMS)oxy],-TMS ester	2.03	–	+
2.	7.46	Benzene, 1-ethyl-3-methyl-	1.69	–	+
3.	9.11	Ethanedioic acid, bis(TMS)ester	3.77	–	+
4.	11.88	Pentadecane	8.63	+	–
5.	13.43	Benzeneacetic acid, TMS ester	12.65	+	–
6.	13.72	Butenedioic acid, bis (TMS)ester	11.45	–	+
7.	17.12	4-Pyridone-MonoTMS	8.63	+	–
8.	17.57,	Docosane	66.12	+	–
9.	19.36	Benzoic acid, 5-methyl-2-TMSoxy-TMS ester	1.02	+	–
10.	21.55	Pyrazine, 2,5-dimethyl-3-propyl	2.03	+	–
11.	22.08	2-Acetoxytetradecane	5.69	+	–
12.	22.63	3,4-Tetramethylene-5-5-pentamethyle-2-nepyrazoline	9.12	+	–
13.	23.31	Tetradecanoic acid, ethyl ester	10.12	+	–
14.	23.74	5-Octadecenal	87.22		–
15.	23.46	Octadecane	16.12	+	–
16.	24.24	Tetradecanoic acid, TMS	1.01	+	–
17.	25.21	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1.09	+	–
18.	25.52	1-Hexadecanol	2.56	+	–
19.	26.65	Hexadecanoic acid, ethyl ester	94.47	+	–
20.	27.47	Dotriacontane	6.41	+	+
21.	28.10	Hexadecanoic acid, TMS ester	84.56	–	+
22.	28.65	1-Octadecanol	12.55	+	
23.	29.70	Òctadecanoic acid, ethyl ester	82.44	+	–
24.	30.36	Òctadecanoic acid, TMS	86.99	+	–
25.	31.33	1-Eicosanol	8.01	+	–
26.	31.46	Heptacosane	1.86	–	+
27.	31.92	Hentricontane	1.02	+	–
28.	32.15	Methyl 19-methyl-eicosanoate	7.84	+	–
29.	32.14	Pentacosane	17.69	–	+
30.	32.57	Docosane	67.89	–	+
31.	33.40	1-Docosanol, acetate	12.07	+	–
32.	33.98	Hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester	11.25	+	–
33.	34.20	1,2-Benzenedicarboxylic acid, diisooctyl ester	13.01	–	+
34.	34.22	Ethyl Docosanoate	9.87	+	–
35.	35.12	2-Tetracosanol, acetate	6.74	+	–
36.	35.29	2-Monostearin, TMS ether	66.55	+	–
37.	34.50	Cleidoniol, deoxy	2.56	–	+
38.	35.57	Octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester	1.20	+	–
39.	35.72	Ethyl tetracosanoate	6.41	+	–
40.	35.90	1-Heptatricotanol	96.12	–	–
41.	36.11	2,6,10,14,18,22-Tetracohexaene, 2,6,10,15,19,23-hexamethyl	45.75	–	+
42.	36.81	17-Pentatricontene	11.46	+	
43.	37.09	Nonacosane	12.67	–	+
44.	37.22	Oleic acid, 3-(octadecyclooxy)propyl ester	7.88	+	–
45.	37.58	Octacosanic acid	5.69	+	–
46.	37.72	15,17, 19,21-Hexatriacontatrayne	4.21	+	–
47.	37.98	Heptacosane	1.26	+	–
48.	40.12	Silane,[[(3β,22E)-ergosta-7,22-dien-3-yl]trimethyl	12.24	–	+
49.	42.49	Silane, trimethyl[[(3β,5α)-stigmastan-3-yl]oxy]-	8.46	–	–
50.	44.41	β-sitosterol TMS ether	7.26	–	+
51.	48.29	9,12-Octadecadienoic acid (Z,Z)-, 2,3-bis[(TMS)oxy]propyl ester	1.06	–	–

(+) present; (−) absent; RT: Retention time; UTDE: Untreated distillery effluent; BTDE: Bacterial treated distillery effluent; TMS: Trimethylsilyl.

By contrast to the above, the bacterial degraded BMDE showed improvement in seed germination and seedling growth parameters compared to their untreated forms as illustrated in Fig. 6 and Table 3. The results of the phytotoxicity assay presented in Table 3, showed that the phytotoxicity of distillery effluent was reduced pointedly after treatment by VKDS9. The significant increase in seed germination percentage was recorded up to 100% at tested BMDE as compared to the values obtained with untreated effluent, whereas no germination was recorded at 50% concentration of BMDE. In the present study, the seedling length was increased by 4.7, 4.1, 3.5, 2.3, and 1.3 cm for 10, 20, 30, 40, and 50%, respectively in response to exposure to bacterial treated effluent. In all examined seeds, shoot and root length gradually increased with increasing concentrations of bacterial treated BMDE compared to untreated effluent. An increase in percentage germination and promotion of seedling growth in bacterial treated BMDE at higher (50%) concentrations suggested reduction of toxicity of metabolic products formed after bacterial treatment (Table 3). The reduced toxicity of metabolites creates a favorable environmental condition for germinating seeds and seedling growth in the tested sample. The study revealed that the *C. arientinum* L. seeds exposing to bacterial treated 10 and 20% BMDE showed 85.45 and 74.54 germination recorded, respectively. Chowdhary et al. (2020) demonstrated that after microbial remediation, some

Table 3
Evaluation of physiological parameters of seed germination and seedling growth in *Cicer arientinum* L. irrigated with untreated and bacterial treated biomethanated distillery effluent.

Treatment	No. of seed	RL (cm)	RSG (%)	GI (%)	RRG (%)	PI (%)	PP (%)	SVI	TI	SL	RSR	SMR
Untreated Distillery Effluent												
10%	10	3.9 ± 0.21	100 ± 1.00	70.90 ± 1.26	70.90 ± 0.15	1.6 ± 0.00	29.09 ± 0.09	660 ± 112	0.70 ± 0.00	2.70 ± 0.08	1.44 ± 0.12	0.00 ± 0.00
20%	10	3.5 ± 0.02	90 ± 0.50	57.20 ± 1.19	63.63 ± 0.26	2.0 ± 0.00	36.36 ± 0.42	468 ± 105	0.63 ± 0.00	1.70 ± 0.06	2.05 ± 0.35	1.00 ± 0.01
30%	10	1.5 ± 0.01	80 ± 0.20	21.18 ± 0.06	27.27 ± 0.74	4.0 ± 0.00	72.72 ± 0.34	800 ± 123	0.27 ± 0.00	0.00 ± 0.00	0.00 ± 0.32	2.00 ± 0.25
40%	10	1.0 ± 0.00	60 ± 0.10	10.90 ± 0.21	18.18 ± 0.06	0.0 ± 0.00	81.81 ± 0.66	600 ± 124	0.18 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 0.65
50%	10	NG	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	NG	0.00 ± 0.00	10.00 ± 2.12
Bacterial Treated Distillery Effluent												
10%	10	4.7 ±0.45[a]	100 ±0.55[a]	85.45 ±1.24[b]	85.45 ±1.22[b]	0.8 ±0.00[c]	14.54 ±0.24[a]	780 ± 136[a]	0.85 ±0.00[c]	3.1 ±0.21[c]	1.51 ±0.08[c]	0.00 ±0.00[ns]
20%	10	4.1 ±0.32[a]	100 ±0.23[a]	74.54 ±1.64[b]	74.54 ±1.01[c]	1.4 ±0.00[c]	25.45 ±0.46[a]	700 ± 121[a]	0.74 ±0.00[c]	2.9 ±0.36[c]	1.41 ±0.04[b]	0.00 ±0.00[ns]
30%	10	3.5 ±0.21[a]	100 ±0.12[a]	63.60 ±0.97[b]	63.63 ±1.26[a]	2.0 ±0.00[ns]	36.36 ±0.21[a]	580 ± 118[a]	0.63 ±0.00[c]	2.3 ±0.48[c]	1.52 ±0.07[a]	0.00 ±0.00[ns]
40%	10	2.3 ±0.41[b]	100 ±0.11[a]	41.80 ±0.64[a]	41.81 ±0.46[a]	3.2 ±0.00[ns]	58.18 ±0.16[a]	330 ± 109[a]	0.41 ±0.00[c]	1.0 ±0.66[c]	2.3 ±0.42[a]	0.00 ±0.00[ns]
50%	10	1.3 ±0.02[b]	100 ±0.25[a]	23.63 ±0.02[a]	23.63 ±0.21[a]	4.2 ±0.00[c]	76.36 ±0.98[a]	130 ± 110[a]	0.23 ±0.0[a]	0.00 ±0.00[ns]	0.00 ±0.00[ns]	0.00 ±0.00[ns]
Tap Water	10	5.5 ± 0.00	100 ± 1.00	100 ± 0.00	100 ± 1.23	0.00 ± 0.00	NT	880 ± 121	1.00 ± 0.03	3.3 ± 0.65	1.66 ± 0.04	0.00 ± 0.00

All values are mean (n = 3) ± S.D., RSG: Relative seed germination; RL: Root length; GI: Germination index; RRE: Relative root growth; RRG: Relative root germination; PI: phytotoxicity index; PP: Phytotoxicity percentage; DI: Delayed index; SVI: Seed vigor index; TI: Tolerance index; SL: Shoot length; RSR: Root shoot ratio; SMR: Seedling mortality rate; NT = No toxicity was observed; NG = No growth or germination was observed. All the values are mean ±SD. (n = 3); Students *t* test (two tailed as compared to untreated effluent); [c]Less significant at $p < 0.05$; [ns]Non significant at $p > 0.05$; [a]Highly significant at $p < 0.001$; [b]Significant at $p < 0.01$.

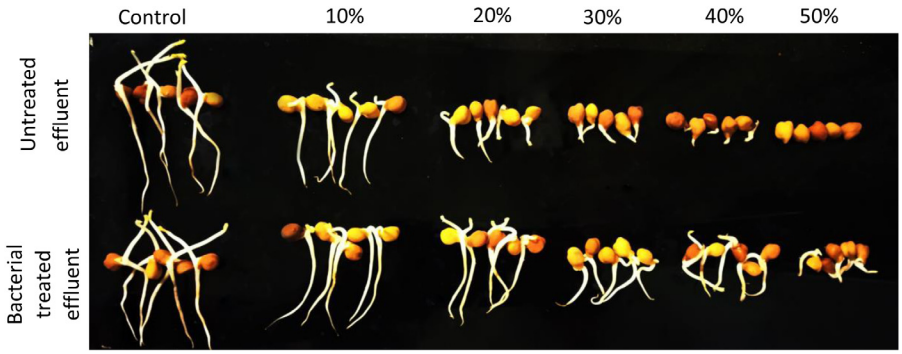

Fig. 6. Effect of untreated and bacterial treated biomethanated distillery effluent on germination and seedling growth of *Cicer arientinum* L. Control was used as a distilled water.

effluent products were less toxic in some cases. The enhance germination percentage and growth seedling of seeds after biodegradation of distillery effluent by microorganism has been reported by Bharagava and Chandra (2010)

4. Conclusion

The current study revealed that the discharged BMDE contained elevated TSS, TDS, BOD_5, COD, organometallic complex, and metallic elements which pose a risk to the ecosystem. Results also demonstrated the high potential of *B. albus* strain VKDS9, isolated from degraded distillery sludge, which accelerated reduction of color up to 83% in existence of adequate carbon–nitrogen sources under a wide range of abiotic conditions, with pH (6.0–8.0), temperature (25–45 °C), and agitation (150–200 rpm) within 144 h of incubation by activities of laccase, MnP and LiP. The metabolites analysis by spectroscopic techniques (HPLC and GC–MS) showed the degradation of BMDE by potent bacterial strain. The phytotoxicity evaluation using seed of *C. arientinum* L. further confirmed reduction of BMDE toxicity after biotreatment. This is the first information evaluating the potential of *B. albus* strain VKDS9 under static conditions for the biodegradation and detoxification of BMDE. In the light of the above finding, it is concluded that isolated bacteria could be a suitable candidate for the development of bioremediation technologies used for in situ degradation and detoxification of effluent released by the distilleries.

CRediT authorship contribution statement

Vineet Kumar: Conceptualization, Validation, Methodology, Investigation, Software, Visualization, Supervision, Writing – original draft, Review & editing. **Sakshi Agrawal:** Investigation, Methodology, Validation, Analysis. **Sushil Kumar Shahi:** Conceptualization, Resources, Visualization, Formal analysis. **Ankit Motghare:** Conceptualization, Validation, Methodology, Investigation, Software, Visualization. **Simranjeet Singh:** Visualization, Software, Investigation, Validation. **Praveen C. Ramamurthy:** Resources, Validation, Software, Visualization, Writing – review & editing.

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.

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Appendix A. Supplementary data

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