Title: Optimizing the metribuzin degrading potential of a novel bacterial consortium based on Taguchi design of experiment

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Abstract

Metribuzin (MB) is used for control of weeds in crops like potato, maize and sugarcane. Its extensive and unjudicial use has resulted in various environmental issues; hence it is very critical to remediate this herbicide at the respective point source. Plant associated, MB degrading bacterial strains, *Rhodococcus rhodochrous* sp. AQ1, *Bacillus tequilensis* sp. AQ2, *Bacillus aryabhattai* sp. AQ3 and *Bacillus safensis* sp. AQ4 were isolated, and a consortium MB3R was developed. For degradation of MB by the consortium MB3R, various parameters i.e., pH, temperature, inoculum density and pesticide concentration were optimized by using Taguchi design of experiment (DOE). MB degradation was dependent upon all the four factors. The contribution of each factor on MB degradation was according to the order: temperature > inoculum density > pH > pesticide concentration. Fitness of Taguchi DOE in forecasting the optimum response, was confirmed experimentally by using optimized levels of the four factors i.e., pH 7.0, temperature 30 °C, pesticide concentration 45 mg l^{-1} and an inoculum density of 5.0×10^{5} CFU ml^{-1} whereby 98.63 % MB degradation was observed. Appearance and subsequent degradation of three MB metabolites, desamino-metribuzin (DA), diketo-metribuzin (DK) and desamino-diketo-metribuzin (DADK) during biodegradation by the consortium was observed.

Keywords: Metribuzin, Taguchi DOE, diketo-metribuzin (DK), desamino-metribuzin (DA), desamino-diketo metribuzin (DADK)

1 Introduction

Metribuzin (4-amino-6-tert-4, 5-dihydro-3-methylthio-1, 2, 4-triazin- 5-one, MB) is a selective triazinone herbicide, used for the pre- and post-emergence treatment of many grasses and broadleaf weeds in various crops such as potato, sugarcane, maize, and tomato. It kills the susceptible plant by pairing with one of the proteins of photosystem II complex and hence inhibits the process of photosynthesis [1].

Metribuzin is highly soluble (1.05 g l^{-1}) in water, an average K_{oc} of 53.13 which varies with soil types and a K_{ow} of 1.70. High water solubility and weak sorption of MB to soil increase threat of the surface as well as ground water contamination by the seepage of residual MB. Ground and surface water contamination by MB have been reported by many authors [2-5]. Spray and vapour drift, runoff and leaching from treated land, or from accidental spills can cause contamination of water with MB. MB is highly toxic to freshwater macrophytes and algae [6]. It can also cause physiological disorders in higher animals by disrupting the endocrine system [7] and is thus included in the list of regulated endocrine disruptors in many countries. In addition, MB residues can disturb the crop rotation systems by affecting the establishment of subsequent crops [8]. Soil microbial populations and their activities are also affected by the residues of MB [9].

The environmental and health problems associated with herbicide contamination arouse attention towards its remediation in eco-friendly and cost-effective way, at point source i.e. before they enter the water bodies and environment. Different bioremediation strategies are used as an effective, safe, and cheap way to clean up contaminated environments [10] whereby activities of living organism (plants and/or microorganisms and their enzymes) are used for the decomposition and transformation of specific contaminants into less toxic or inactive elements [11]. Soil microbiota have the ability to take part actively in the transformation of herbicides [12, 13]. Several bacteria able to degrade triazine herbicides have been isolated [14, 15]. Major focus of the studies was degradation of atrazine, the most widely used triazine herbicide. Only few microbes capable of MB degradation have been reported so far [16,17]. Zhang *et al.,* (2014) reported *Bacillus* sp. N1, that can degrade 73.5 % of 20 mg l^{-1} MB within 5 days at pH 7.0 and 30 °C and Gopal *et al.,* (2011) reported *Burkholderia cepacia* CH9 that could degrade 86.0 % of 50 mg l^{-1} MB (within 20 days) [18, 19].

For successful bioremediation, the presence of specific inocula and conditions such as incubation temperature, pH, pesticide concentrations, inoculum size for their growth and

contaminant degradation are important factors [20]. Thus, it is valuable to explore and optimize various parameters (media components and environmental factors) that have an impact on biodegradation of MB [21, 22]. The optimization of one factor at a time while keeping all others constant is time consuming, laborious and also does not provide any information about the interactive effects of the tested factors [23]. Hence, it is imperative to use statistically designed experimental approaches e.g., Taguchi design of experiment (DOE) that provide information on direct as well as interactive effects of all variable factors at the same time. Taguchi DOE uses signal-to-noise (S/N) ratio for the analysis of all the experiments and is helpful for the easy estimation of optimal combination of factors [24].

The present study was designed to isolate and characterize novel, MB degrading bacteria for the development of a bacterial consortium to be used for bioremediation of this herbicide and to optimize degradation condition by using Taguchi DOE. In addition, the kinetic parameters of MB biodegradation by the isolated bacterial strains and their consortium MB3R were also studied. Metabolites produced during MB biodegradation were identified by GC/MS. This is a pioneer report for use of Taguchi design of experiment for the optimization of MB degradation by a bacterial consortium.

2 Material and Methods

2.1 Chemicals

Analytical grade MB (99.4 %) purchased from Sigma-Aldrich (USA) was used as a standard. Technical grade MB (97 %) used in this study was obtained from Four Brothers Chemicals, Lahore, Pakistan. The analytical grade standards of metribuzin-desamino (DA), metribuzin-diketo (DK) and metribuzin- desamino- diketo (DADK) were procured from Dr. Ehrenstorfer GmbH (Germany). Dichloromethane, acetonitrile and methanol (HPLC and LC-MS/GC-MS grade) were purchased from Merck. All other chemicals used were purchased from Sigma-Aldrich, Merck or BDH.

2.2 Enrichment, isolation and screening of MB degrading bacteria

For the enrichment of MB degrading bacteria, soil samples were collected from potato vegetated field at Arifwala, Pakistan, where this herbicide had been sprayed repeatedly. The soil was further spiked with MB and sludge. Potato seeds were sown in this herbicide-amended soil in pots in triplicate. Twelve weeks after sowing, potato plants were harvested and rhizospheric as well as endophytic bacteria were isolated by the following procedures.

For the isolation of rhizospheric bacteria, wet rhizosphere soil having 20-30 % moisture was sampled from each pot separately. The rhizospheric MB degrading bacteria

were isolated by following the procedure reported by Anwar *et al*., [25]. Briefly about 20 g soil was added into 250 ml Erlenmeyer flasks having 100 ml minimal salt medium (MSM) and 50 mg l^{-1} MB, incubated at 100 rpm and 30 °C for one week, 5 ml culture from each flask was recovered and transferred into fresh MSM having same concentration of MB successively up to four weeks. Ten-fold dilutions of cultures were prepared and 100 µL of each dilution was spread on LB agar plates containing 50 mg l^{-1} MB. After 2-3 days of incubation, morphologically different single bacterial colonies were picked and streaked repeatedly onto LB agar plates until purified colonies were obtained.

For the isolation of endophytic MB degrading bacteria, root samples (about 0.5 g) from each plant were taken separately. The roots were washed thoroughly for 10 minutes with tap water to remove attached soil and then surface-sterilized to eliminate epiphytic bacteria by adopting the procedure described by Rashid *et al*., [26]. Briefly, roots were dipped in ethanol (75 %) for 3 min, rinsed 3 times with autoclaved water, soaked for 5 min in sodium hypochlorite solution (2.5 %, w/v) and rinsed 5 times with sterile water. The efficiency of surface sterilization was checked by placing 100 µl aliquots of the final rinsing water on Lysogeny-Broth (LB) solid medium and incubated at 30 °C for 2 days. After surface sterilization, the roots were crushed aseptically with sterile pestle and mortar into a paste by adding 1-3 ml autoclaved distilled water. Serial dilutions (10^{-1} to 10^{-5}) from each root paste were prepared and spread onto separate LB agar plates containing 50 mg l^{-1} MB and incubated at 30 °C in an incubator. After 24 hrs, bacterial growth was checked with the naked eye as well as under a stereoscope. Morphologically distinct single colonies were streaked repeatedly onto LB agar plates containing MB until isolated purified colonies were obtained.

MB utilization capability of these isolates was monitored by streaking them on MSM agar plates at (25, 50 and 100 mg l^{-1}) as sole source of carbon. The proficient isolates were further screened by culturing in minimal salt medium (MSM) containing MB at varying concentrations.

2.3 Identification of the bacterial isolates

The universal primers 27f (5′ AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′ GGTTACCTTGTTACGACTT-3′) were used for the amplification of the 16S rRNA gene [25]. The sequences of the amplicons obtained were used to identify the strains using NCBI BLAST, ClustalX 1.8.1 and MEGA 6.06.

2.4 Preparation of the bacterial consortium MB3R

For the preparation of the bacterial consortium, strains *Rhodococcus rhodochrous* sp. AQ1, *Bacillus tequilensis* sp. AQ2, *Bacillus aryabhattai* sp. AQ3 and *Bacillus safensis* sp. AQ4 were grown individually in liquid LB medium for 24 hrs and centrifuged at 5000 × g for 10 minutes to prepare cell pellets. The pellets were washed and suspended in sterile saline (0.85 % NaCl) to set an optical density (OD) of 1.0 at 590 nm. The strains were mixed in a 1:1:1:1 proportion to form bacterial consortium MB3R [27].

2.5 MB degradation capability of individual strains and the consortium MB3R

A laboratory-scale shake flask experiment using strains *Rhodococcus rhodochrous* sp. AQ1, *Bacillus tequilensis* sp. AQ2, *Bacillus aryabhattai* sp. AQ3, *Bacillus safensis* sp. AQ4 as well as the consortium MB3R was conducted to record MB degradation. The cultures were grown in individual 250 ml Erlenmeyer flasks containing 100 ml MSM with 25 mg l^{-1} MB as sole carbon source and incubated on a rotary shaker at 100 rpm and 30 °C. The experiment was replicated thrice and uninoculated flasks were used as control. Representative samples were taken at 0 (just after inoculation), 4, 8, 12 and 15 days after incubation (DAI). The OD and residual MB concentration of each sample was measured by spectrophotometer at 590 nm and HPLC, respectively. The viability of all the bacterial strains in the culture media was confirmed by streaking on LB agar plates.

Kinetics parameters for MB biodegradation were determined by plotting ln $[C_t/C_0]$ versus time (days). Equation 1 and 2 were used for the determination of the degradation rate constant (k, d^{-1}) and half-life ($T_{1/2}$, d) correspondingly.

$$C_t = C_0 \times e^{-kt} \qquad \text{Eq. (1)}$$

$$T_{1/2} = \ln(2)/k \qquad \text{Eq. (2)}$$

Where C_t represents concentration of MB (mg l^{-1}) at time "t" and C_0 represents concentration of MB (mg l^{-1}) at time "zero".

2.6 Optimization of MB biodegradation conditions by Taguchi DOE

The Taguchi DOE was employed to optimize various physical and chemical culture conditions by orthogonal arrays. The robust design aids to improve the process of optimization by reducing influence of noise factors [28].

Initially, multiple experiments were conducted for selection and optimization of various factors that have substantial influence on the MB biodegradation. An experiment was arranged by L9 orthogonal array scheme from Taguchi optimization method using Minitab and Qualiteck-4 software for the optimization of MB biodegradation by the bacterial

consortium MB3R. The four-selected factors pH, temperature, initial pesticide concentration, and initial inoculum size (Table 1) were arranged in L9 orthogonal array (Table 2). After 15 days of incubation, HPLC analysis was performed to quantify the residual MB. The data thus obtained was analyzed using Qualitek-4 software (Nutek Inc., MI, USA) to identify the individual as well interactive effect of factors and optimal conditions for MB removal on the basis of signal to noise ratio (S/N ratio) with the option "bigger is better." The S/N ratio was calculated by the following formula:

$$S/N = 10*\log (S (1/Y^2)/n) \quad \text{Eq. (3)}$$

Where "Y" represents the % biodegradation (response) from certain factor level combination while "n" denotes the number of responses in this combination.

2.7 HPLC analysis of MB

For extraction of MB, samples (10 ml) recovered from culture flasks were centrifuged at 7200 × g for 10 min to obtain cell free medium. An equal volume of dichloromethane (DCM) was used twice to extract MB from the supernatant. Organic layers of DCM were aspirated, pooled and evaporated at room temperature under nitrogen. The residues were dissolved in HPLC grade acetonitrile (1 ml) and then filtered through fluorophore™ filter membrane (0.45 m FH) to remove any particles [25]. These extracts were subjected to HPLC analysis (PerkinElmer HPLC) by using reverse-phase ODS2 C18 column and isocratic elution with acetonitrile: water (80:20) acidified with acetic acid (0.5 %) as mobile phase at a flow rate of 1 ml min^{-1}. The retention time of the MB was 2.39 min at 280 nm wavelength.

2.8 Identification of degradation metabolites by GC-MS

The metabolites produced during the biodegradation of MB were identified by Gas Chromatography-Mass Spectrometry (GC-MS). For this purpose, the bacterial consortium MB3R was inoculated in MSM supplemented with MB at 25 mg l^{-1} and incubated on a rotary shaker at 100 rpm and 30 °C. After 0, 4, 8 and 12 days of incubation, representative samples were taken, extracted with dichloromethane (DCM), liquified in acetonitrile (LC-MS grade) and filtered through PTFE 0.45 μm membrane filter.

These extracted samples were run on a GC-MS machine (6890 GC-5973N MSD, Agilent) for the identification of known MB metabolites. The conditions of the instrument were as follows: initial temperature was 60 °C (2 min); thereafter, it increased to 280 °C (5 min) at 12 °C min^{-1}. The injector temperature was 280 °C, and the helium flow was 1.0 ml min^{-1}. The corresponding compounds were identified by the interpretation samples mass spectra NIST mass spectral search program (version 2.0).

3 Results

3.1 Isolation and screening of MB degrading bacteria

Initially, 15 morphologically different isolates were isolated on LB agar plates containing 50 mg l^{-1} MB. To test the ability of these isolates to utilize MB as sole carbon and energy source, their growth was monitored on MSM agar plates containing 25, 50 and 100 mg l^{-1} MB. Eight of the 15 isolates were able to grow well on MSM agar plates. In each case, highest number of colonies appeared on the plates containing 25 mg l^{-1} MB followed by 50 mg l^{-1}. In contrast, few colonies appeared on the plates containing 100 mg l^{-1} MB. For further selection of promising MB degrading bacteria, these isolates were cultured individually in liquid MSM containing 50 mg l^{-1} MB. After one week of incubation, residual MB was quantified and % degradation was calculated. On the basis of degradation efficiency, four isolates AQ1, AQ2, AQ3 and AQ4, capable to utilize 78, 61, 67 and 58 % MB, respectively were selected for further studies. When the concentration of the metribuzin exceeded 50 mg l^{-1}, bacterial growth as indicated by increase in OD and herbicide removal was slower upto 90 mg l^{-1}. At 100 mg l^{-1}, there was no bacterial growth. Potentially, higher MB concentration showed inhibitory effect on the growth of these bacteria.

3.2 Identification of bacterial isolates

The 16S rRNA gene sequences of bacterial isolates AQ1, AQ2, AQ3 and AQ4 showed 99% identity with corresponding gene sequences of *Rhodococcus rhodochrous* strain 372, *Bacillus tequilensis* strain 10b, *Bacillus aryabhattai* strain B8W22 and *Bacillus safensis* NBRC 100820, respectively (Fig. 1). The assigned GenBank accession numbers of the four strains are given in parenthesis: *R. rhodochrous* sp. AQ1 (MG966499), *B. tequilensis* sp. AQ2 (MG966500), *B. aryabhattai* sp. AQ3 (MG966501), and *B. safensis* sp. AQ4 (MG966502).

3.3 MB biodegradation by pure cultures and the consortium MB3R

At 25 mg l^{-1} initial concentration, the consortium MB3R utilized 95.61 % of the added MB at 16 DAI compared to 89.23, 77.93, 82.71, 75.14 % MB utilized by the pure cultures of *R. rhodochrous* sp. AQ1, *B. tequilensis* sp. AQ2, *B. aryabhattai* sp. AQ3 and *B. safensis* sp. AQ4 respectively at 16 DAI (Table 3). The bacterial consortium MB3R degraded MB significantly faster than the individual strains as indicated by higher K(d^{-1}) i.e. degradation rate constant (Table 4). All four constituent strains of MB3R were present in the culture media by the end of experiment (Table 1, supplementary material) indicating their stability, growth and involvement in the degradation of MB.

Plots of ln (C_t/C_0) of residual MB versus incubation days revealed that MB degradation followed first order kinetics (Fig. 2). The MB3R culture also showed significantly lower $T_{1/2}$ (half-lives) as compared to the axenic cultures. Thus, MB3R was selected for further MB degradation studies.

3.4 Optimization of MB biodegradation based on Taguchi design of experiment

3.4.1 Designed matrix experimentation

The influence of four factors i.e., pH, temperature, pesticide concentrations, and inoculum density on the biodegradation of MB was optimized by using L9 orthogonal array, Taguchi DOE. The experiment No. 3 (pH, 6.0; temperature, 35 °C; pesticide concentrations, 45 mg l^{-1} and inoculum density, 5×10^5 CFU ml^{-1}) resulted in maximum MB degradation (93.83 %) as well as S/N ratio (39.48) while minimum MB degradation (31.36 %) and S/N ratio (29.86) were observed for experiment No. 1 (Table 2).

3.4.2 Effect of independent factors on MB biodegradation

The results showed that the MB degradation depends upon all the selected factors (Table 5). The difference between S/N ratio at two levels (L2-L1/L3-L1) of each factor indicates its comparative effect, the influence is stronger if the difference is higher. Temperature and pH showed maximum influence on MB degradation at level 2 as compared to pesticides concentration and inoculum size where maximum influence was observed at level 3. The relative contribution of each factor on performance of MB degradation was according to the order: temperature > inoculum density > pH > pesticide concentration (Fig. 3).

Individual effects of all the selected factors, at various levels on MB biodegradation by MB3R are depicted in Fig. 4. An increase in temperature from 25 °C (L1) to 30 °C (L2) increased the S/N ratio to maximum. Similar trend was observed for pH where maximum S/N ratio was observed at level 2 (pH 7). For inoculum density and pesticide concentration, highest S/N ratio was observed at level 3 followed by level 2 and level 1.

3.4.3 Interactive influence of factors on MB biodegradation

The severity index (SI) calculated from the interactions of factors at various levels is presented in Table 2 (supplementary materials). The results indicated that pH and pesticide concentration interact with each other at level 1 and 3 to give a maximum SI value (54.46 %) that is followed by the interaction of pH and inoculum density at level 1 and 3 (SI of 42.77 %). The lowest SI was observed between temperature and inoculum density (14.3 %).

3.4.4 Analysis of variance (ANOVA)

Due to the interactions of significant factors and their levels in varying combinations, maximum biodegradation is achieved. Analysis of variance (Table 6) depicted that all selected factors and their interactions in the experimental design were statistically significant at 95 % confidence level as based on the F ratio. ANOVA exhibited that temperature had maximum effect on the degradation of MB while the inoculum density and pH had minor contributions and the pesticide concentration was the least contributing factor.

3.4.5 Optimization and validation of factors

Optimum conditions for MB biodegradation and performance of optimized factors as determined by Taguchi DOE are presented in Table 7 and Fig. 5 respectively. Temperature was observed as the most important factor for MB degradation followed by inoculum density, pesticide concentration and media pH. As predicted by the Taguchi DOE, 97.56 % MB degradation can be attained by employing optimized levels of different factors i.e., media pH 7.0 (level 2), temperature 30 °C (level 2), pesticide concentration 45 mg l^{-1} (level 3) and an inoculum density of 5.0×10^{5} CFU ml^{-1} (level 3).

An experiment was conducted to confirm the fitness of Taguchi orthogonal array in forecasting the optimum response, using predicted optimum conditions. Observed MB degradation was 98.63 % which significantly similar to the predicted value (97.56 %) at 95 % level of confidence (data not shown).

3.5 MB metabolites produced during biodegradation

To identify metabolites produced during MB biodegradation, extracts of MB3R cultures were analyzed by GC-MS. A single peak at 16.991 min having m/z 214 was observed in the total ion chromatogram (TIC) of the samples collected at zero time. Fragmentation ions of this peak matched with the mass spectrum (MS) of the authentic MB standard. Peaks at retention times 16.693, 15.126 and 13.278 min with m/z 199,184 and 169, respectively, were found in the TIC of samples collected at 4, 8 and 12 DAI (Fig. 6). These peaks were identified desamino-metribuzin (DA), diketo-metribuzin (DK) and desamino-diketo-metribuzin (DADK) by comparing their mass spectrum, retention times and fragmentation patterns with respective authentic standards.

There was a gradual decrease in MB concentration until 12 days DAI. At 4 DAI, all the three metabolites were detected. Concentration of DA further decreased at 8 and 12 DAI whereas concentration of DK and DADK increased at 8 and decreased at 12 DAI. Varying concentrations of metribuzin oxidation products, DA and DK during biodegradation as well

as gradual decrease in DADK concentration indicate the appearance and subsequent degradation of all the three metabolites of MB.

4 Discussion

In this study, four plant associated bacterial strains, *R. rhodochrous* sp. AQ1, *B. tequilensis* sp. AQ2, *B. aryabhattai* sp. AQ3 and *B. safensis* sp. AQ4 capable of utilizing MB as a source of carbon were obtained. MB degrading bacteria were enriched in the rhizo- and endosphere of plants growing under the stress of high MB concentration and isolated thereafter. Potentially, plants growing in MB contaminated soil positively affected the proliferation of MB degrading bacteria both in the rhizo-and endosphere and hindered growth of the bacteria sensitive to this herbicide. Three of the isolates were gram-positive, endospore forming *Bacillus* spp. and one was gram negative *Rhodococcus* sp. A consortium MB3R comprising of these bacterial strains was more effective for degradation of MB as compared to the individual strains. To date, only two bacterial strains *Bacillus* sp. N1 and *Burkholderia cepacia* CH9 capable to degrade MB in liquid cultures have been reported in independent studies [18, 19]. Degradation potential of the consortium reported in the current study is higher (95.6 % degradation) as compared to previous reports whereby *Bacillus* sp. N1 and *Burkholderia cepacia* CH9 degraded only 73.5 and 86 % of the added MB respectively. Higher MB degradation potential of the consortium could be because of the interactions among bacterial strains belonging to different *Bacillus* spp. and *Rhodococcus* sp. for their survival and growth under the stress caused by the presence of herbicide. Moreover, it has been reported that bacterial consortia exhibit potential to co-metabolize noxious xenobiotics efficiently in contrast to single bacterial strains [29, 30]. Potentially, different types of bacteria in the consortium express a variety of enzymes to boost the degradation of pollutants [31]. Previously, researchers have characterized bacterial consortia for the effective and improved degradation of persistent organic pollutants belonging to diverse chemical classes including phenyl urea herbicide diuron [32], chlorpyrifos [33], atrazine [34], profenfos [22] and bispyribac sodium [27].

The bioremediation approach is employed to enhance the process of biodegradation taking place naturally either by inoculation of the contaminant degrading bacteria into the polluted environments (bioaugmentation) or by enhancing the biodegradation potential of indigenous bacteria through the optimization of environmental conditions (biostimulation) [35]. The environmental and culture conditions are very critical for the success of bioremediation process [36] as the composition, structure and metabolic capabilities of soil

microbial inhabitants mainly depend on them [37]. To address this issue, various environmental parameters (temperature, pH) and culture conditions (pesticide concentration and inoculum density) were optimized by employing Taguchi DOE to enhance the biodegradation of MB by the consortium MB3R.

In Taguchi DOE, multiple factors are optimized simultaneously, which helps to reduce time and energy required to design and conduct the experiments and analyze the results thus obtained [24]. This approach is also supportive for investigation of significant interactions between different parameters by reducing influence of noise factors [38]. Many researchers [24, 39, 40] have employed Taguchi DOE for optimization of various physiochemical parameters to enhance the efficiency of contaminant biodegradation. To date, no report is available about the use of a statistical model to optimize biodegradation of MB by bacteria. Among the combinations of different factors (L9 orthogonal array), maximum MB degradation was obtained at pH, 6.0; temperature, 35 °C; pesticide concentrations, 45 mg l^{-1} and inoculum density, 5×10^5 CFU ml^{-1}.

Based on experimental results, Qualitek-4 software calculates S/N ratio which determines the individual effect of various levels of each factor, higher S/N ratio being the better. As indicated by S/N ratio, optimum temperature for MB degradation by the consortium was 30 °C (level 2) whereas at higher and lower temperatures (level 1 and level 3), MB degradation was lower. This might be due to the inhibitory effect of low or higher temperature on bacterial growth and enzymatic / metabolic activities [18]. Higher S/N at pH 7.0 (level 2) as compared to pH 6.0 (level 1) and 8.0 (level 3) demonstrated that the consortium performed better at neutral pH. Role of media pH to affect enzymatic activities, bacterial growth and metabolism is well documented [41]. For inoculum size, highest S/N ratio was obtained at 5.0×10^5 CFU ml^{-1} (level 3) followed by levels 2 and 1 respectively. S/N ratio decreased gradually with decreasing inoculum size, potentially because a threshold level of bacteria in medium would be required for degradation to occur.

During degradation process, any of the controllable factor interacts with other factors. In Taguchi DOE, the severity index (SI) which represents interactions among factors helps to better understand and optimize such processes. In the present studies, most significant interactions were found between media pH (level 1) and pesticide concentration (level 3) followed by media pH (level 1) and inoculum density (level 3).

The analysis of variance (ANOVA) explains the significance of different factors on the basis of P-value and F-ratio. ANOVA indicated that temperature and inoculum size are

the most important factor affecting MB biodegradation by MB3R with relatively less influence of media pH and pesticide concentration.

As predicted by the Taguchi DOE, 97.56 % MB degradation can be attained by employing optimized levels of different factors i.e., media pH (level 2), temperature (level 2), pesticide concentration (level 3) and an inoculum density (level 3). In an experiment conducted, using predicted optimum conditions, 98.63 % MB degradation was observed which is significantly similar to the predicted value (97.56 %) at 95 % level of confidence (Data not shown). Hence, on the basis of results obtained, it can be concluded that Taguchi DOE would be a good approach for the optimization of different culture conditions.

The metribuzin degradation pathway is presented in the Fig.1 (supplementary material). An analysis of GC-MS spectral data revealed that three metabolites DA, DK and DADK were produced during biodegradation of MB by the consortium MB3R. The appearance and subsequent degradation of metabolites DA, DK as well as DADK, were associated with the disappearance of metribuzin. Apparently, oxidation by MB3R first resulted in the deamination or oxidative desulfuration of metribuzin. During deamination, amino group from the MB ring is detached with the formation of DA [42, 43] while, DK is formed by oxidative desulfuration of methylthio group of metribuzin [44]. The continuous oxidation of DA and DK removes the remaining methylthio and amino groups respectively, with the formation of DADK [45]. In case of complete degradation of metribuzin, DADK is further transformed into water and CO_2 [46]. The results obtained in this study depicted a higher concentration of DK in solution than DA, suggesting some preference for the oxidative desulfuration over deamination pathway [47]. Moreover, further degradation of DADK was also anticipated from the gradual decrease in the its peak intensity.

5 Conclusions

A consortium MB3R comprising of MB degrading bacterial strains was found to be more effective for biodegradation of the herbicide as compared to axenic cultures. Using Taguchi DOE, optimized levels of various factors i.e., pH, temperature, pesticide concentration, and inoculation density were obtained which helped to achieve enhanced MB degradation by the consortium MB3R. Metribuzin biodegradation was found to be associated with its disappearance, transient accumulation of the metabolites desamino-metribuzin (DA), diketo-metribuzin (DK) and desamino-diketo-metribuzin (DADK) together with concomitant increase in bacterial biomass in culture media where MB was the only carbon source.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 2: L9 Orthogonal array generated by Taguchi design of experiment presenting combinations of factors and experimental results as response (% biodegradation)

Exp. No.	Factors Levels				Response (% biodegradation)				S/N ratio
	pH	T	PC	ID	R1	R2	R3	average	
1	L1	L1	L1	L1	28.9	30.7	34.5	31.36	29.859
2	L1	L2	L2	L2	77.4	76.3	74.9	76.20	37.636
3	L1	L3	L3	L3	91.8	92.5	97.2	93.83	39.438
4	L2	L1	L2	L3	58.1	58.5	52.8	55.80	34.911
5	L2	L2	L3	L1	76.3	82.1	79.9	79.43	37.988
6	L2	L3	L1	L2	72.1	76.8	78.3	75.73	37.569
7	L3	L1	L3	L2	40.8	38.9	43.6	41.20	32.248
8	L3	L2	L1	L3	68.1	66.9	71.3	68.76	36.738
9	L3	L3	L2	L1	56.3	53.1	50.2	53.20	34.489

S/N ratio = signal to noise ratio

Table 3: Degradation of metribuzin (%) by individual strains and bacterial consortium MB3R over time

Strains	0 DAI	4 DAI	8 DAI	12 DAI	16 DAI
Control	1.0	1.58	2.88	3.46	3.94
Rhodococcus rhodochrous sp. AQ1	0.6	49.11	71.50	82.03	89.23
Bacillus tequilensis sp. AQ2	1.2	36.80	59.51	69.27	77.93
Bacillus aryabhattai sp. AQ3	0.8	40.90	66.85	76.27	82.71
Bacillus safensis sp. AQ4	1.0	36.00	58.00	71.49	75.14
Bacterial consortium MB3R	0.6	59.75	75.86	87.43	95.61

DAI = days after incubation

R^2= regression coefficient

Table 5: Effects of individual factors on MB degradation based on S/N ratio

Sr no.	Factors	Level 1	Level 2	Level 3	L2-L1	L3-L1
1	Media pH	35.645	36.823	34.492	1.177	-1.115
2	Temperature	32.339	37.454	37.166	5.115	4.826
3	Pesticide concentration	34.722	35.679	36.558	0.957	1.835
4	Inoculum density	34.112	35.818	37.029	1.705	2.917

Table 6: Analysis of variance (ANOVA)

Sr. No.	Factors	DOF (f)	Sum of sqres. (S)	Variance (V)	F-ratio (F)	Pure Sum (S')	Percent P (%)
1	Media pH	2	1279.38	639.69	87.68	1264.79	12.97
2	Temperature	2	6045.47	3022.74	414.34	6030.38	61.86
3	Pesticide concentration	2	811.20	405.60	55.60	796.61	8.17
4	Inoculum density	2	1482.17	741.08	101.58	1467.58	15.05
5	Other errors	18	131.32	7.30			1.98
	Total	26	75.63				100.00

DOF= degree of freedom

Figure

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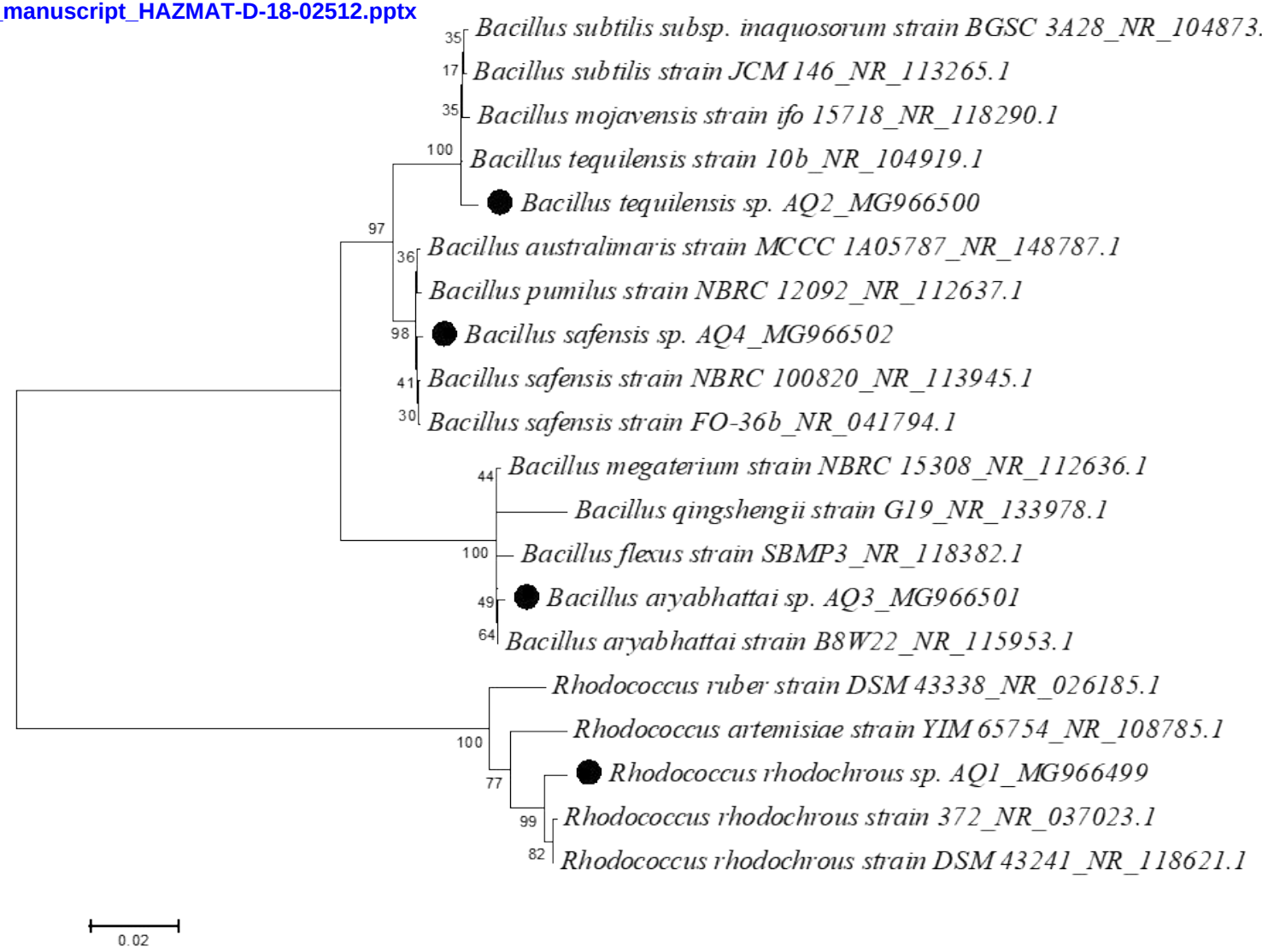
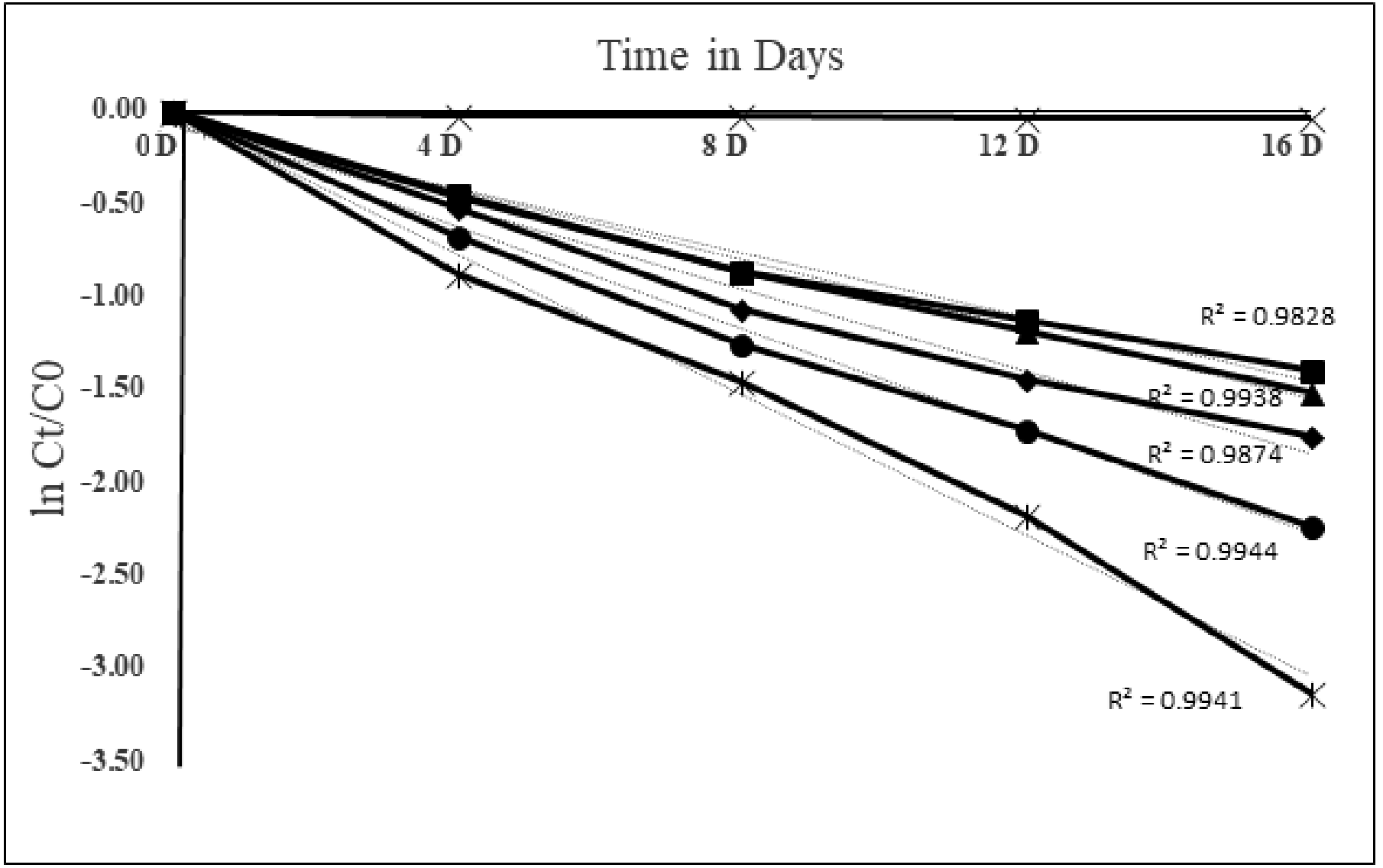


Fig. 1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ln Ct/C0
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4 D
8 D
12 D
16 D
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R² = 0.9938
R² = 0.9874
R² = 0.9944
R² = 0.9941

Fig. 2

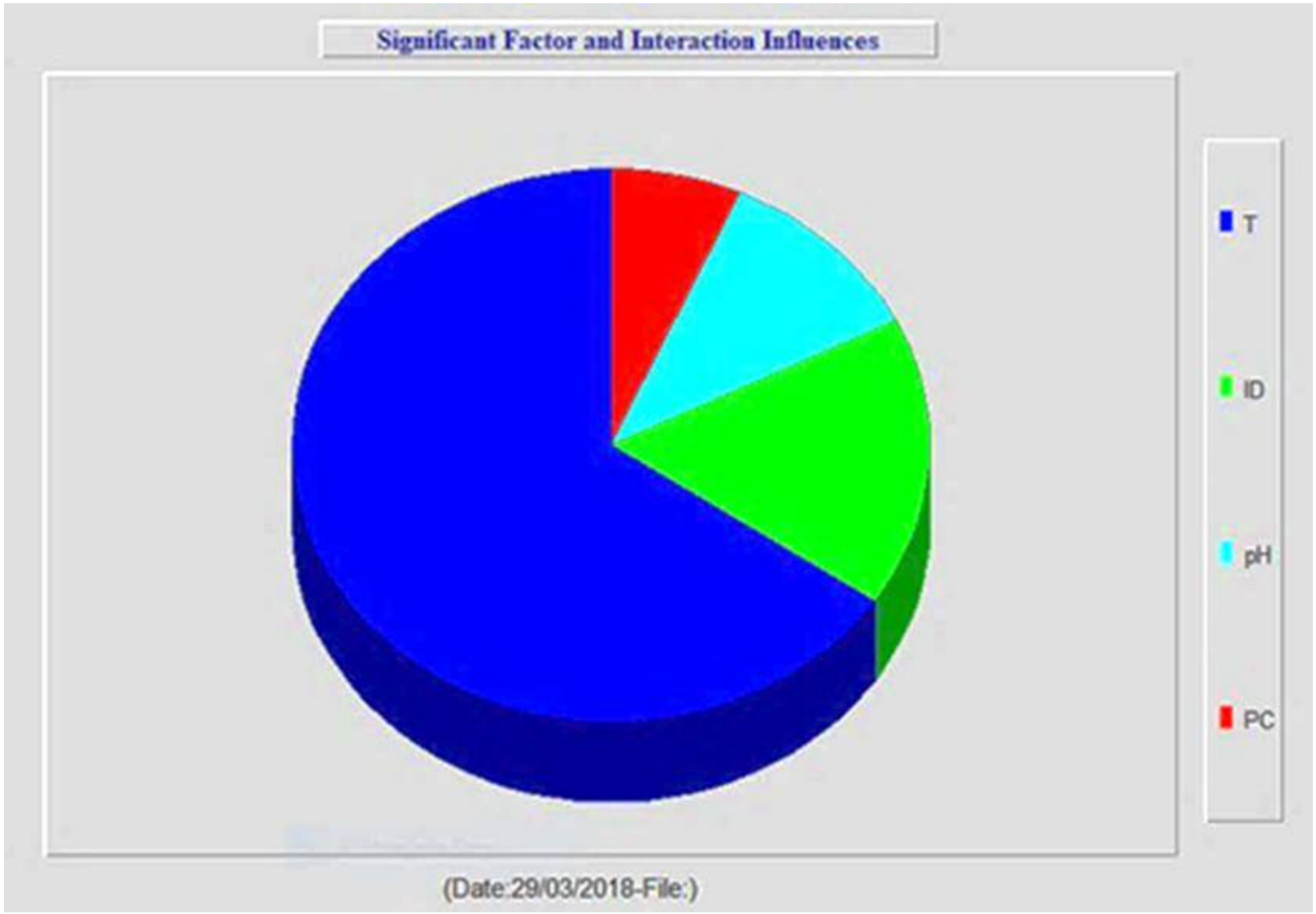
Significant Factor and Interaction Influences
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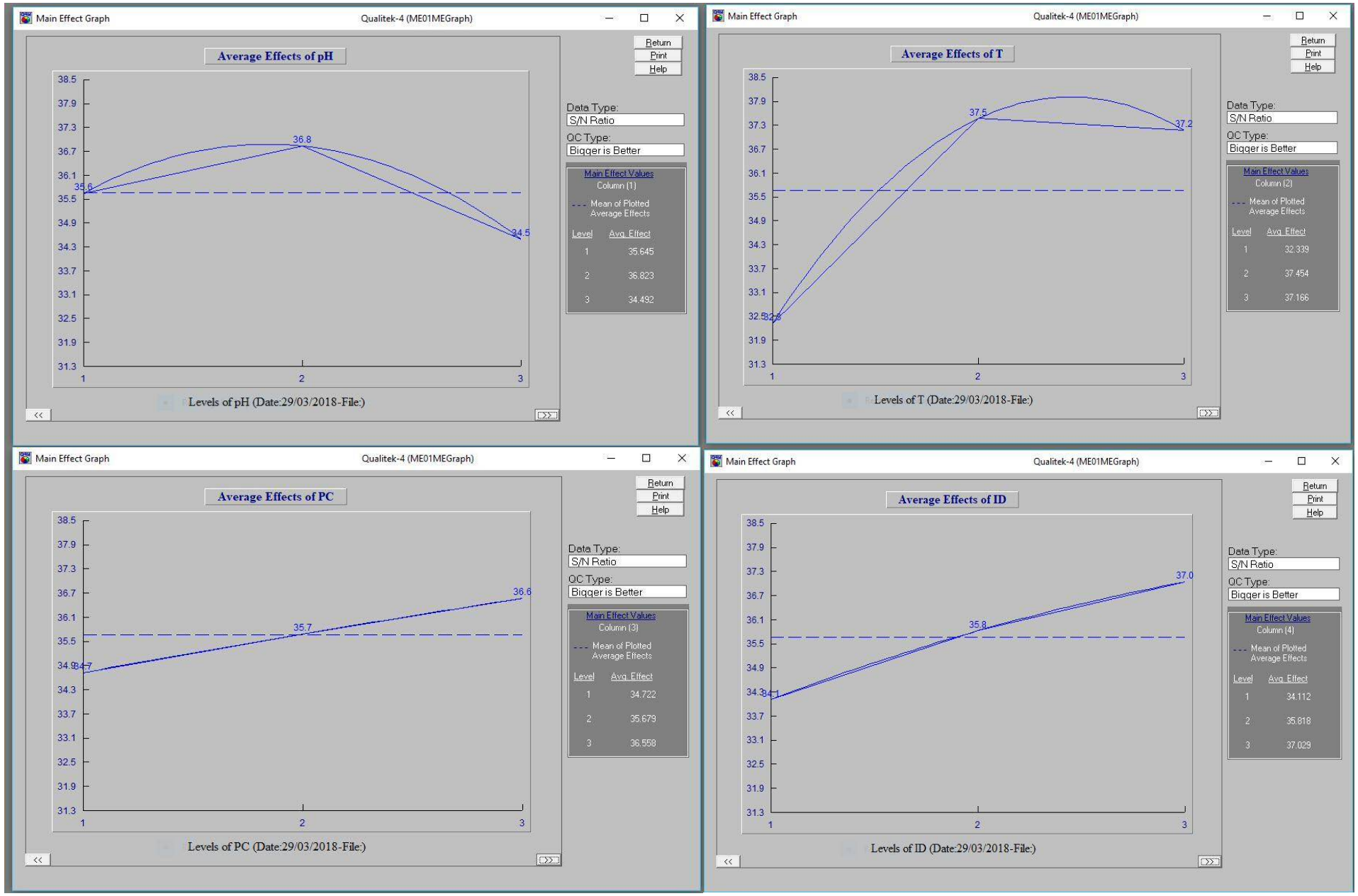
Fig. 3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Average Effects of pH
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Main Effect Values
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Mean of Plotted Average Effects
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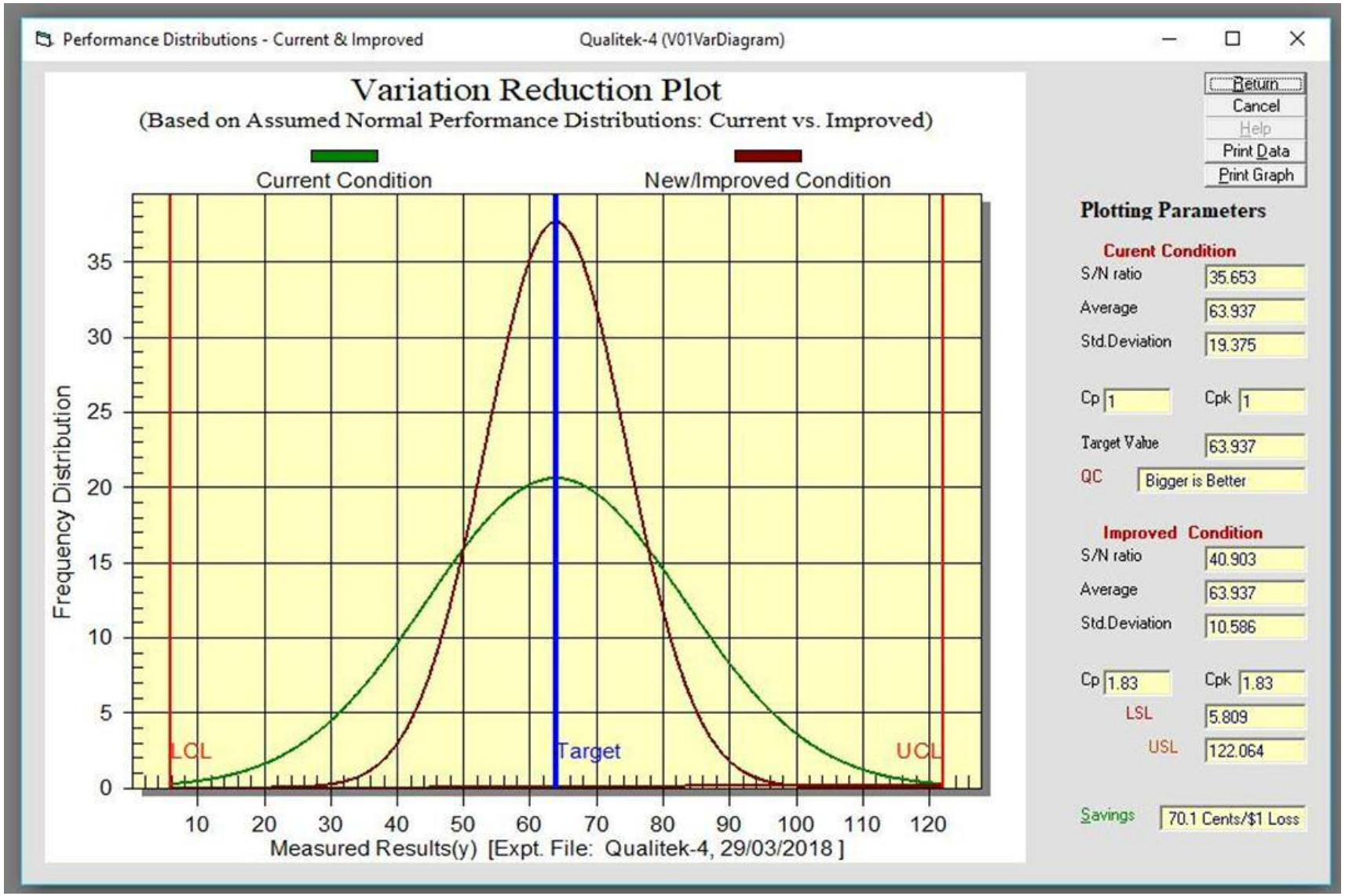
Fig. 4


Performance Distributions - Current & Improved
Qualitek-4 (V01VarDiagram)
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(Based on Assumed Normal Performance Distributions: Current vs. Improved)
Current Condition
New/Improved Condition
Frequency Distribution
Measured Results(y) [Expt. File: Qualitek-4, 29/03/2018]
LCL
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Curent Condition
S/N ratio 35.653
Average 63.937
Std.Deviation 19.375
Cp 1
Cpk 1
Target Value 63.937
QC Bigger is Better
Improved Condition
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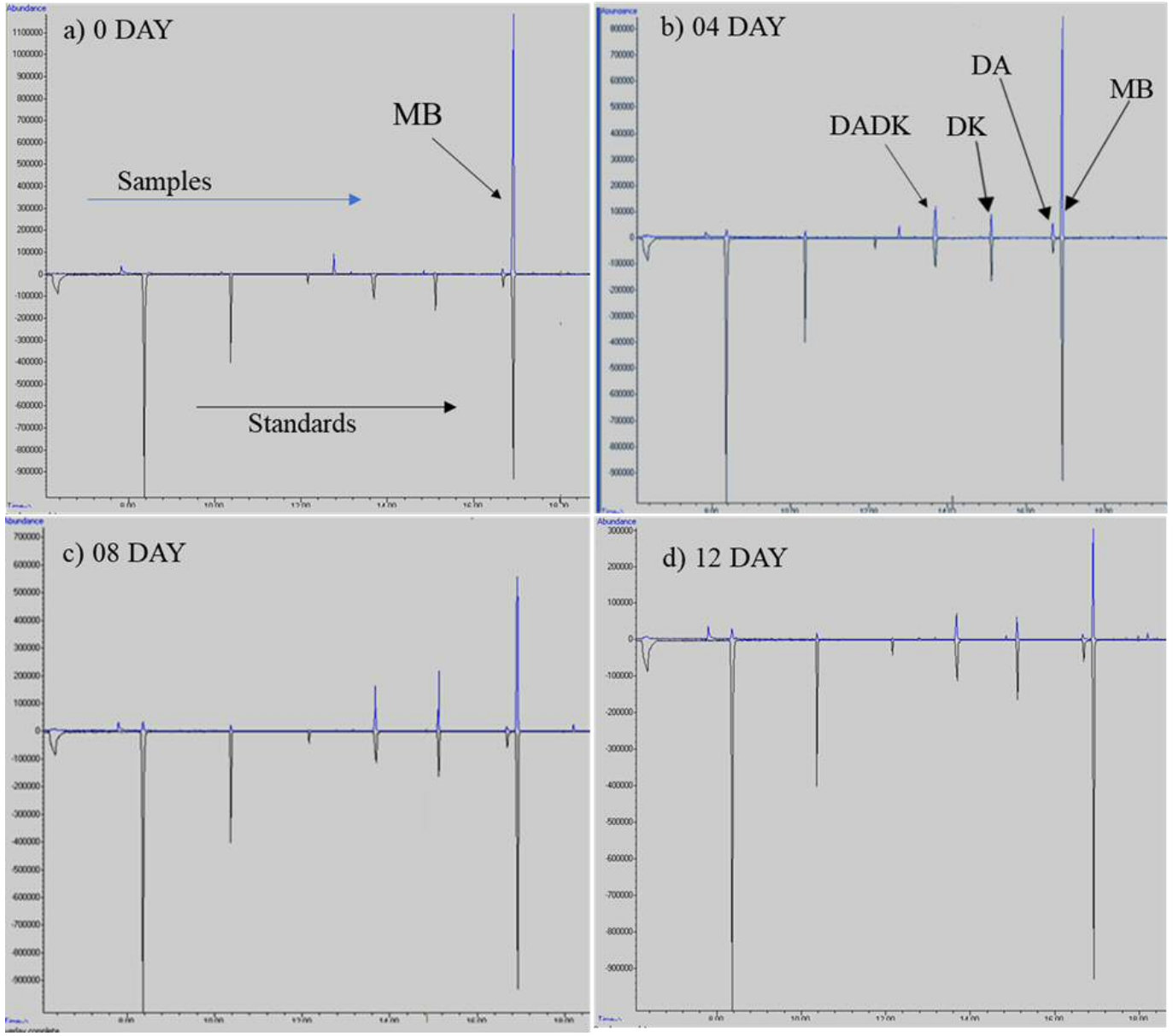
Fig. 5


a) 0 DAY
MB
Samples
Standards
b) 04 DAY
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DA
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c) 08 DAY
d) 12 DAY

Fig. 6

Figure captions

Fig 1: Phylogenetic tree constructed using 16S rRNA gene sequences of strains AQ1, AQ2, AQ3, AQ4 and the closely related reference strains. Tree was constructed using MEGA6 software with 1000 bootstrap. Bootstrap values are given at each branch node. Accession numbers of the reference type strain are presented.

Fig. 2: Semi logarithmic plot of C_t/C_0 presenting biodegradation of metribuzin by *Rhodococcus rhodochrous* sp. AQ1 (●), *Bacillus tequilensis* sp. AQ2 (▲), *Bacillus aryabhattai* sp. AQ3 (♦), *Bacillus safensis* sp. AQ4 (■) and the consortium MB3R in MSM at 50 mg L^{-1} initial concentration. Dotted lines show the experimental data whereas solid line depicts the first order fit.

Fig. 3: Comparative influence of significant factors and their interactions on biodegradation of metribuzin by MB3R. Factor T (temperature) contributes maximum, covering a large area in the figure while PC (pesticide concentration) imparts little contribution in the biodegradation process.

Fig. 4: Individual effect of all the selected factors pH, temperature (T), pesticide concentration (PC) and inoculum density (ID) at various levels on the biodegradation of metribuzin by MB3R.

Fig. 5: Showing variation reduction plot based on assumed normal performance distribution of current vs improved condition.

Fig. 6: Mass spectrum of metribuzin and its metabolites i.e., desamino-metribuzin (DA), diketo-metribuzin (DK) and desamino-diketo-metribuzin(DADK) in samples (upward) extracted at 0 day (a), 4^{th} day (b), 8^{th} day (c) and 12^{th} day (d) after incubation compared with analytical standards (downward).

Supplementary Material

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