RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2025, **15**, 37461

Biodegradation mechanism of butralin by a newly isolated *Bacillus velezensis* FC02

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Butralin is a selective pre-budding agent. It is relatively immobile in the substrate after application, and its transmission through the food chain poses a potential threat to human health. In this study, we isolated a butralin-degrading strain FC02, and identified it as *Bacillus velezensis*. Optimization of butralin degradation with Box–Behnken design resulted in 89.12% degradation at pH 8.4, OD_{600} = 2.5, 35.0 °C, and 200 mg FC02. Strain FC02 efficiently converted butralin into five products through nitro-reduction, dealkylation, and hydroxylation reactions, differing from the mechanisms and pathways in other strains. Genomics and transcriptomic analyses revealed that nitroreductase genes are involved in the nitro-reduction process of butralin degradation. Here, we explored the degradation characteristics and metabolic pathways of strain FC02, providing an in-depth understanding of the mechanism of microbiological degradation of butralin.

Received 27th March 2025
Accepted 27th September 2025

DOI: 10.1039/d5ra02138c

rsc.li/rsc-advances

Introduction

Butralin (*N-sec*-Butyl-4-(2-methyl-2-propanyl)-2,6-dinitroanilin) is a selective pre-budding agent used to inhibit axillary tobacco bud growth.[1] Butralin functions by reducing plant cell differentiations and division.[2] When it enters the plant through the hypocotyl of the monocots root and the dicotyledones root or its hooked process, butralin impairs cell mitosis and blocks root and bud growth, ultimately leading to lodging, twisting, growth arrest, and plant death.[3] As the effective tobacco bud suppressant and herbicide butralin has been utilized for controlling both tobacco armpit bud growth and weeds during cultivation.[4,5]

Butralin causes environmental pollution, both directly and indirectly, harming various organisms, including land and aquatic animals and beneficial insects, as well as humans through the food chain.[6] Unlike many dinitroaniline herbicides, butralin shows no appreciable migration in soil, and the resulting accumulation can irreversibly inhibit soil respiration. Based on an environmental risk assessment, it is slowly degraded mainly through abiotic conditions such as volatilization and light. Additionally, butralin shows high toxicity toward freshwater invertebrates, and inhalation of butralin preparations or dust can cause severe eyes, skin, and respiratory tract irritation.[7] For instance, Refaie and co-workers[8] reported that female rats treat with mild (30 mg kg^{-1} body weight per day) and high (300 mg kg^{-1} body weight per day) concentrations of butralin experienced liver damage and weight loss due to butralin toxicity. Liao *et al.* found butralin in 100 tobacco samples in Fuzhou City, with levels between 15.8 and 499.1 μg kg^{-1},[9] which exceeded the maximum recommended limit (5.0 mg kg^{-1}) set by the China Tobacco Monopoly Administration in 2002.

Common methods mainly including photodegradation, adsorption, bioremediation and so on for reducing, the butralin content in agricultural products and the environment.[10] Currently, bioremediation based on microorganisms is the primary method utilized for organic pollutant removal. The degradation or detoxification of pesticides by microorganisms has become a research hotspot worldwide.[11–14] Research has shown that degrading microorganisms from the genera *Bacillus*, *Pseudomonas*, *Penicillium*, *Aspergillus*, and *Fusarium* have strong degradation effects on dinitroaniline herbicides. The application of butralin to soil can promote the growth of some microorganisms and have a bactericidal effect on others. Butralin markedly affects the activity of soil urease, with increased activity seen after treatment with butralin.[15] However, after a period of adaptation to high pesticide concentrations, the soil microorganisms showed some tolerance to butralin. However, there are few reports on microbial degradation of butralin. Ni and colleagues[16] isolated and purified *Bacillus* spp. Y3, a strain capable of degrading butralin in activated sludge from polluted wastewater; Ghatge Sunil isolated and purified *Sphingopyxis* sp. HMH from the soil in the Korean agricultural area of Qingshan, can effectively degrade butralin.[17] Wang *et al.* reported that a new strain *Bacillus* sp. LY05 can degrade butralin.[1] LY05 was immobilized on tobacco stem charcoal, together with sodium alginate, and

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a chitosan carrier, rapidly degrading butralin in tobacco leaves. The degradation pathways employed by the aforementioned bacterial strains are relatively limited, predominantly relying on nitro-reduction as the principal metabolic mechanism. The mechanisms utilized by these butralin-degrading strains have not been fully elucidated, and further investigation is required to explore the complete degradation characteristics and degradation products of butralin by the purified bacteria.

This study aimed to screen and isolate strain FC02, was isolated from the unique ecological niche of the shipwreck, highly effective for butralin degradation and identify factors influencing the biodegradation of butralin, potential biodegradation products and biodegradation genes. Clarifying the multi-reaction synergy degradation process and mechanism of butralin in the environment can provide an effective means to reduce butralin residues in soil, water, and crops and is of great significance in evaluating the environmental behavior, toxicology, and ecological security of butralin after application, providing technical support for ensuring the safety of agricultural products and the farmland environment.

Experimental

Chemicals and solutions

Butralin (98.7% purity) was purchased from Beijing Castermo Technology Development Co., Ltd. The acetonitrile and methanol were purchased from Tianjin Yifang Technology Co., Ltd (China). Butralin was dissolved in methanolto yield a stock solution of 5000 g L^{-1} and was kept in a brown bottle at 4 °C. Mineral salt medium (MSM), NA, and LB media were used to cultivate a butralin-degrading microbial consortium.[18]

Enrichment, screening, and purification of the butralin biodegrading strain

Squared wood samples of 1 cm^2 were collected from the sunken ship "Nanhai I", placed in a conical flask containing 100 mL LB medium with a concentration 50 mg L^{-1} of butralin, and placed in a shaker at 28 °C at 200 pm for one week. The culture was continued for two weeks, inoculated with 10% (v/v) of the inoculum of the grown culture in new enriched medium supplemented with increasing concentrations of butralin (100 mg L^{-1} for week 1 and 200 mg L^{-1} for week 2). Then, 10% (v/v) of the culture was inoculated into MSM containing 200 mg L^{-1} butralin, and the culture was continued for one week. One hundred microliter of MSM was spread on an NA solid medium plate and cultured at 28 °C in a constant temperature incubator for 2–3 days.[17,19] Single colonies with different morphological characteristics were selected and inoculated on separation medium containing 200 mg L^{-1} butralin. The streak plate method was used three times for separation and purification, and butralin degradation was assessed by clearance surrounding single colonies and was further evaluated by high-performance liquid chromatography (HPLC) (Waters, USA). An isolate, FC02, that showed the greatest degradation was selected for further study.

The FC02 isolate evaluated in terms of its physiochemical and morphological features, together with its 16S rRNA gene sequence. Morphology was further examined by scanning electron microscopy (SEM) (Thermo Fisher, Waltham, MA, USA) evaluating its the external morphology, size, surface viscosity, transparency, and odor. Physiochemical assays were performed as described in Bergey's Manual of Systematic Bacteriology.[20]

Single factor test on butralin degradation by *Bacillus velezensis* FC02

To determine the influence of the inoculum amount of FC02, environmental temperature, and pH on butralin degradation, we inoculated starter cultures of strain FC02 in MSM at the same temperature (28 °C), and adjusted the cell concentration to make its OD_{600} values of 0.50, 1.0, 1.5, 2.0, 2.5, 3.0, following by the addition of butralin to 100 mg L^{-1}.[21,22] Based on the inoculation method and environmental relevance, as agricultural soils where butralin accumulates generally exhibit pH 5.5–8.5 and temperatures of 15–35 °C during application seasons,[4] starter cultures of FC02 were placed in MSM at different temperatures and the cell concentration was adjusted to OD_{600} 1.0. The influence of pH on butralin degradation was determined as previously mentioned; however, the concentration of butralin was 100 mg L^{-1} different pH values. In addition, a culture medium without bacteria was used as a control, and each sample was treated three times.

Optimization of butralin degradation using strain FC02

The optimal temperature, pH, and inoculum were determined according to the findings of the single factor tests, using the response surface method designed by Behnken.[23,24] In this study, MSM + 200 mg L^{-1} butralin was administered A, B, and C represent the amount of inoculum, temperature, and pH, respectively. Low, medium, and high concentrations were represented by −1, 0, and 1, respectively. The results of the 17 processes were processed using the Design Expert software (version 10) and fitted to eqn (1). Design Expert software generates a 3D response surface. The horizontal design of the test factors was presented in Table S1.

$$Y_i = b_0 + \sum b_i X_i + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 \ldots \quad (1)$$

The mathematical model presented in eqn (1) represents a second-order polynomial response surface equation, where Y_i denotes the response variable (butralin degradation efficiency, %), b_0 signifies the constant term representing the intercept, b_i represents the linear coefficients quantifying the main effects of independent variables (X_i, including OD600 (A), temperature (B), and pH (C)), b_{ij} corresponds to the interaction coefficients between variables X_i and X_j, and b_{ii} indicates the quadratic coefficients accounting for nonlinear effects of each variable.

Butralin biodegradation kinetics

The degradation of butralin at different concentrations was simulated using a first-order kinetic model.[25] Butralin was

incubated under optimal conditions for 17 days, and samples were collected regularly for testing. The initial concentrations of butralin (100, 200, 450, 900, 1800, and 3600 mg L^{-1}) were set as previously reported.[26,27] The biodegradation kinetic equation is as follows:

$$S = S_0 e^{-kt} \quad (2)$$

where: S represents the concentration of substrate, (mg L^{-1}); S_0 indicates the initial concentration of substrate, (mg L^{-1}); e is the natural constant, k is the degradation rate constant, and t is the reaction time (h). The equation for the half-life of the substrate degradation is as follows:

$$t_{1/2} = \ln 2/k.$$

Analysis of degradation products and pathways

The quantitative and qualitative determination of butralin was performed using HPLC.[28,29] The degradation products of butralin were determined using LC-MS/MS, and a putative microbial degradation pathway of butralin was speculated after the product structure was identified by mass spectrum analysis and database.[30] The database used for product identification was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

Whole genome sequencing of strain FC02

For whole genome information analysis test of strain FC02 were performed by the Shanghai Meiji Biomedical Technology Co., Ltd (Shanghai, China). The bacterial genome scan map was assembled using SOAPdenovo2 (V 2.04), and a complete bacterial genome map was constructed using Unicycler to obtain a complete chromosome sequence. Glimmer (V3.02), Glimmert, RNAscan-SEv2.0 (V1.3.1) and Barrnap (version 0.9) were used to predict the assembly of scanned images, the chromosome genome, tRNA in the genome and the rRNA in the genome, respectively. The gene function was annotated according to the FC02 protein sequences relative to the NOG databases.[29]

RNA sequencing and analysis of the transcriptome

Both the RNA sequencing and analyses were conducted by Nuohe Zhiyuan Biotechnology Co., Ltd (Beijing, China). RNA quality and quantity were examined using an RNA Nano 6000 Assay Kit on a Bioanalyzer 2100 (Agilent Technologies, CA, USA). Libraries were established and pooled based on concentrations and data contents and were sequenced on an Illumina NovaSeq 6000 platform. Both the building index of the reference genome and alignment of the clean reads with the reference genome were done in Bowtie2 (2.3.4.3). Based on the comparison results of the FC02 amino acid sequence of strain FC02 with GO, KEGG, COG, and NOG databases, the function of strain FC02 was annotated.

Butralin degradation by strain FC02 in tobacco leaves

The degradation effect experiment of FC02 was tested in the artificial climate chamber. Tobacco (variety K326) plants with 10 fully expanded leaves were used. Firstly, 100 mg kg^{-1} butralin was uniformly sprayed on the surface of tobacco leaves and it is about 5 mL per plant and 100 plants in total. One day later, a half of the plants were sprayed by 1000 mL of strain FC02 (1×10^7 cfu mL^{-1}) and the rest were sprayed by water as the control. After treatment, tobacco leaves were harvested at 1 d, 2 d, 4 d, 6 d, 8 d, and 10 d. The butralin concentration was quantitatively determined by HPLC.[31]

Results and discussion

Isolation and purification of butralin degrading strain FC02

Fig. 1a shows the mechanism of butralin degradation by FC02. The bacterial liquid of FC02 is orange yellow in color (Fig. 1b). In the shake culture test (Fig. 1c), the liquid medium containing butralin was yellow, and the medium degraded by FC02 was reddish brown. As shown in Fig. 1d, the solid medium containing 50 mg L^{-1} of butralin was yellow. After FC02 growth and butralin degradation visible clearance zones appeared around the bacteria. HPLC analysis showed that the RT of the butralin standard was 21.0243 min. Five new compound peaks were detected in the spectrum of the culture solution in which FC02 degraded butralin, together with butralin (Fig. 1e).

Single colonies of FC02 were nearly round, and light yellow, with folds on the surface, a convex middle, and cloud diffusion (Fig. 2a). Transmission electron microscopy (Fig. 2b) and SEM (Fig. 2c) showed that the cell wall (CW) and cell membrane (CM) were closely connected, the cell-matrix was evenly distributed, and the sectional surface was regular and structured. The strain was rod-shaped and complete without a capsule. The 16S rRNA sequence analysis indicated that the strain was highly similar to *Bacillus velezensis* KKLW (CP054714). The phylogenetic tree of strain FC02, constructed using closely related species and strains, is shown in Fig. 2d. The FC02contig sequence has been submitted to GenBank (accession number CP118495). According to the its morphology, biochemical features, and 16S rRNA sequence, FC02 was identified as *Bacillus velezensis*.

Ecological remediation technology using whole strains to degrade pesticides is used to degrade a variety of chemical reagents and materials. The use of microorganisms for remediation is advantageous because they are environmentally friendly, highly efficient, and inexpensive. It is also a hot topic in international and domestic research on pesticide residue treatment technologies.[32–34] Biodegradable methods can be used to eliminate the pollution of pesticides to the environment, such as β-cypermethrin, buprofezin, endosulfan and *et al.*[35–37] This study showed that, *Bacillus velezensis* can efficiently degrade butralin, greatly expanding its pesticide degradation spectrum. Only three strains were found to degrade butralin: *B. subtilis* Y3, *Sphingopyxis* sp., and *Bacillus* sp. LY05. Similar to strain *Sphingopyxis* sp., strain FC02 cannot use butralin as a carbon and energy source; therefore, culturing the bacterium also requires suitable carbon and nitrogen sources.

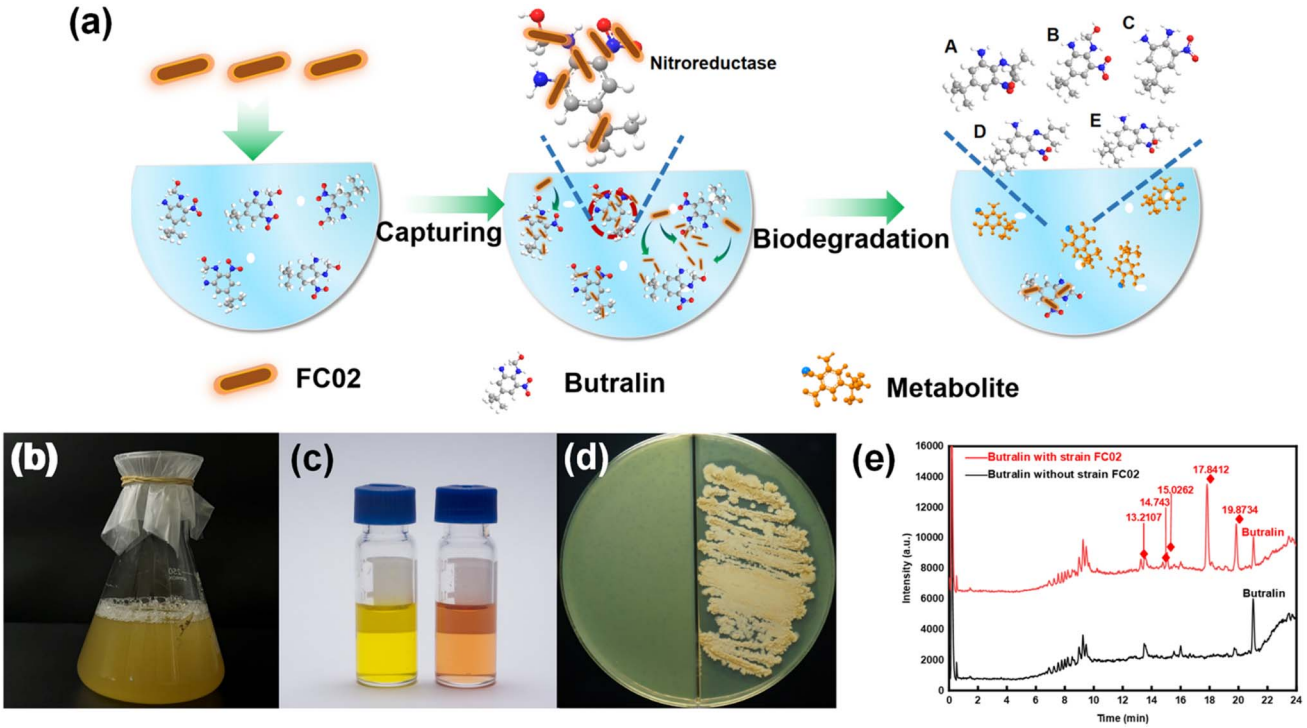

Fig. 1 (a) Schematics of butralin biodegradation by strain FC02; (b) bacterial solution of FC02. (c) The color change of the degradation of butralin by strain FC02 in liquid medium containing butralin. (d) The colonies of FC02 on solid medium containing butralin; (e) HPLC chromatogram of butralin degradation by strain FC02 and resulting metabolites.

The butralin degradation rate of strain FC02 in this study was higher than that of *Bacillus* spp. LY05 and *B. subtilis* Y3.

Single factor test of butralin degradation by strain FC02

The single-factor effect of strain FC02 on butralin degradation is shown in Fig. 3a–c. With an increase in the amount of FC02, the degradation rate of butralin gradually accelerated. When the inoculation amount of OD_{600} was 2–3, more than 90% of butralin was degraded by FC02 within 24 h. At a specific concentration, the degradation rate of butralin was positively correlated with the amount of inoculated FC02 cells. When strain FC02 was incubated at 30–40 °C, the butralin rate was over 90%, and the optimal temperature for butralin degradation was 35 °C, when it reached 97.05% in 24 h. pH variation also affected the degradation rate of butralin. At pH 7.0–9.0, the degradation rate was higher than 90% after 24 h. At pH < 5, the amount of butralin degraded by strain FC02 was less than 60% over the same period. At a pH of 8.0, the degradation rate reached its highest value (96.00%). Univariate degradation assays for strain FC02 helped to determine the independent variable range,[38,39] providing a basis for optimizing butralin degradation.

Optimization of conditions for butralin degradation

Establishment and significance analysis of binary regression model. According to the results of the single-factor test, culture temperature, initial pH, and inoculum size were independent variables in the response surface test.[40] Each factor has three levels (−1, 0, and 1), of which the most appropriate value is the central point of the response surface test. Using the degradation rate of butralin as the response value, a response surface optimization test was designed.[41] The design and results of the Box–Behnken test are presented in Table S2.

Design Expert 10.0.7 was utilized for regression fitting of the data in the table and take the degradation rate of butralin (%) as the response value. The obtained coding equation model is expressed in eqn (3).

$$Y = -789.79 + 214.50 \times A + 31.69 \times B + 7.75 \times C - 0.2550 \times AB - 1.3050 \times AC + 0.1365 \times BC - 38.4030 \times A^2 - 0.4514 \times B^2 - 0.4058 \times C^2 \quad (3)$$

where Y is the degradation rate of butralin (%), A is OD_{600}, B is T (°C), and C is the pH.

The coefficients of the quadratic terms in the equation are all negative, and a single factor indicates that the parabola has a downward opening and a maximum value. Tables S3 and S4 show the outcomes of the variance analysis of the equation model. According to the absolute value of F value in variance analysis, the order of influence of various factors on the degradation of butralin by strain FC02 in this test design is $C > B > A$; that is, pH has the most considerable influence, followed by temperature, and the inoculation amount has little impact on butralin the degradation. In other studies, the inoculum size did not significantly affect the degradation efficiency of microorganisms.[42,43]

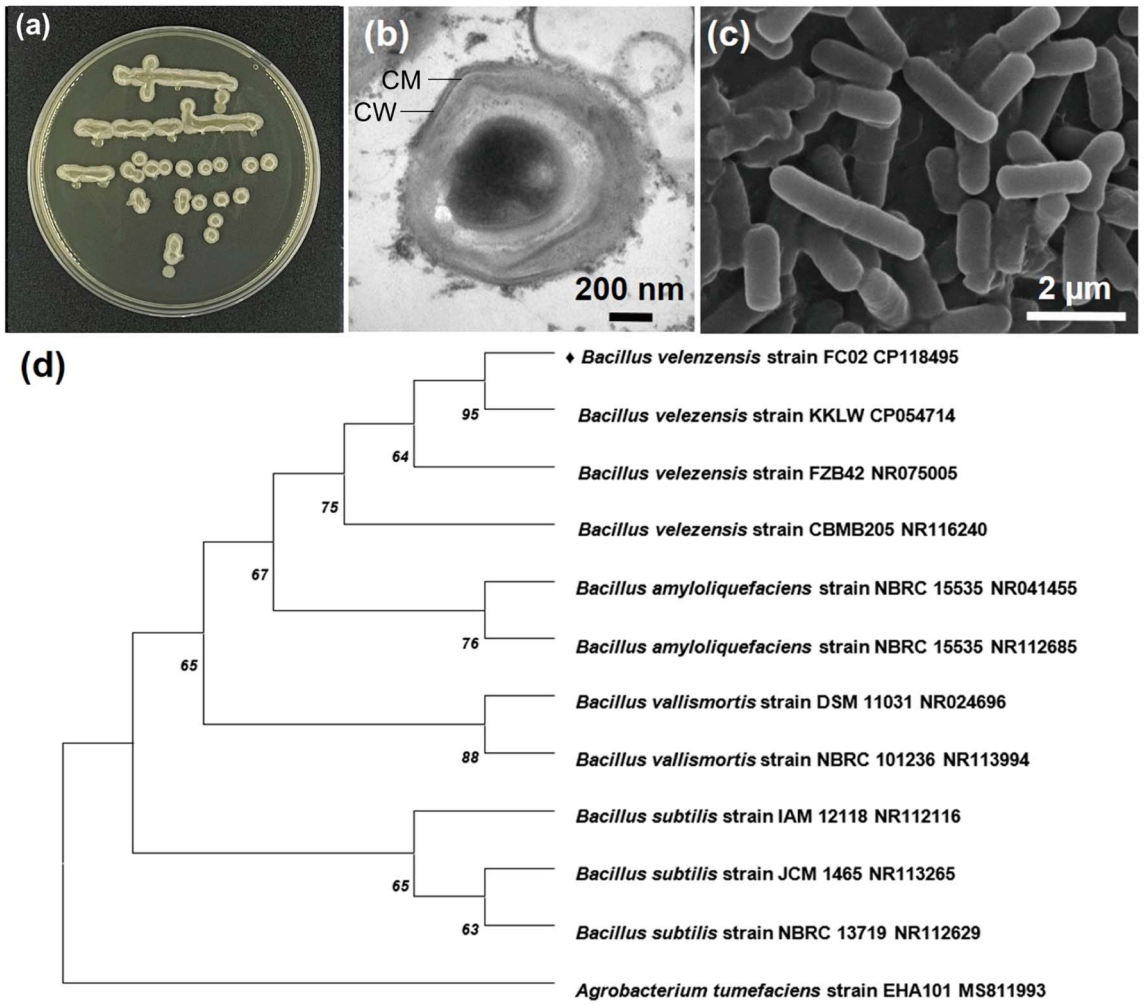

Fig. 2 General aspect of isolated colonies on agar plates (a), transmission electron microscopy (b), and SEM (c) images showing the morphologic characteristics FC02; (d) phylogenetic tree of strain FC02.

Box–Behnken response surface analysis. The response surface curve was compiled using Design Expert 10.0.7 software, and the results are shown in Fig. 3. The model reflects the relationship between inoculum size, pH, temperature, and the degradation ability of strain FC02 and can predict the its butralin degradation activity and efficiency. It can be established from the response surface of the figure that with an increase in inoculum size, temperature, and pH, the degradation rate of butralin first increased and then decreased. The above figure shows that the contour map is oval, indicating that the interactions between inoculum size and temperature, inoculation amount and pH, and temperature and pH were significant.

The optimal conditions for strain FC02 to degrade 200 mg L^{-1} butralin obtained from the model were as follows: $OD_{600} = 2.524$, temperature = 35.389 °C, initial pH = 8.435. The rate of butralin degradation by strain FC02 was expected to be 89.613% within 48 h. To confirm the results obtained from the response surface, the optimal process conditions after correction were OD_{600} = 2.5, 35.0 °C, and initial pH 8.4. The average degradation rate was 89.12% when the experiment was repeated three times under three conditions. The experimental results agreed with theoretical predictions, indicating that the model accurately predicted the degradation of butralin by strain FC02. Strain FC02 is more easily degraded in an alkaline environment, as shown in previous studies.[44–46] The Box–Behnken design of an RSM-based method was not applied to the degradation of butralin, and this study helped determine the optimal degradation conditions of the strains.

Butralin degradation kinetics by FC02

In fitting the first-order kinetic model, the degradation half-life was also consistently delayed with increasing initial butralin concentration. This model fitted the degradation characteristics of the strain well at an initial substrate concentration of 450 mg L^{-1} (Table 1). When the initial amount of substrate exceeded 450 mg L^{-1}, the degradation appeared to have a noticeable lag period, which was delayed with increasing substrate concentration. In the reaction system, the butralin

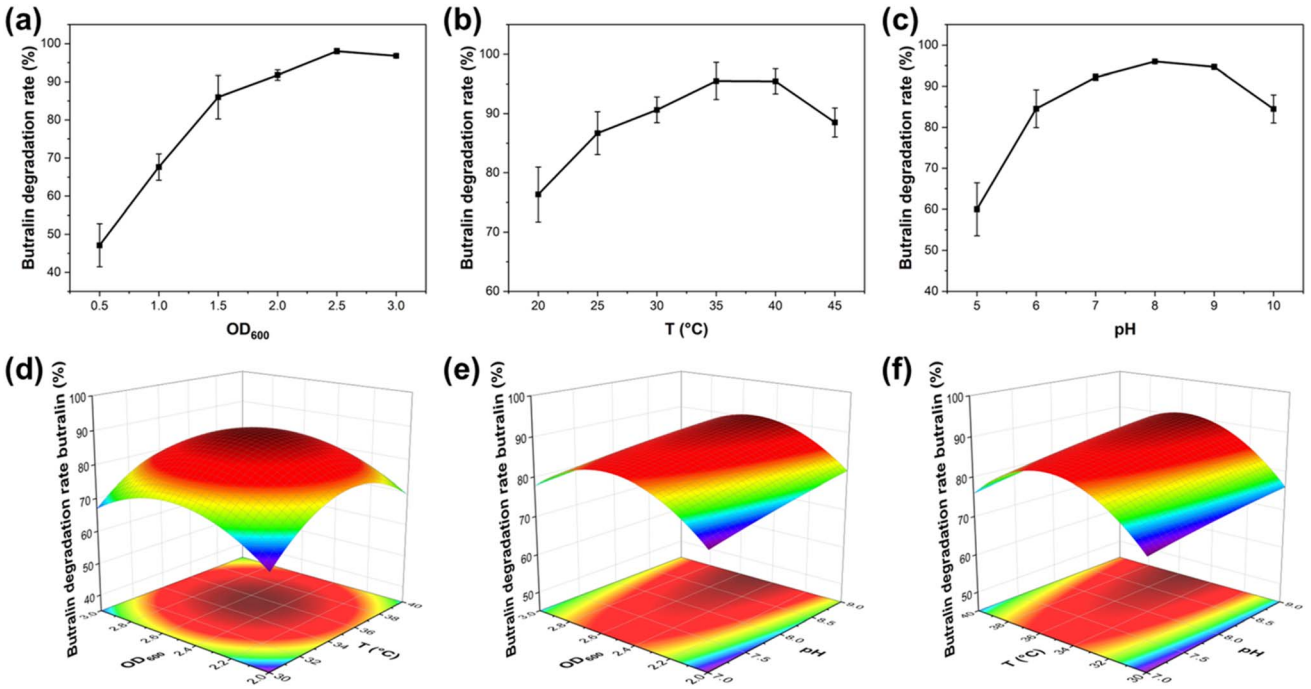

Fig. 3 The influence of OD_{600} (a), temperature (b), and pH (c) on the degradation of butralin by strain FC02; three-dimensional diagrams of butralin degradation by the interactions of OD_{600} and temperature(d), OD_{600} and pH (e), temperature and pH (f).

concentration range was 100–450 mg L^{-1}, the corresponding k was 0.17539–0.18294, and the R^2 value was higher than 0.9. At initial butralin concentrations exceeding 450 mg L^{-1}, the half-life of butralin was prolonged, as shown in Fig. 4. This method has been utilized for assessing the breakdown of various pesticides, including microbial degradation of methylene.[13,47,48] Bacteria capable of degrading high butralin levels have been identified. *Bacillus* sp. LY05 degraded butralin (50 mg L^{-1}) within 72 h with a degradation rate of 73.23%. *Sphingopyxis* sp. HMH effectively degraded 98% of butralin (100 mg L^{-1}). In our study, strain FC02 tolerated and degraded butralin at concentrations up to 3600 mg L^{-1}, but its half-life was much longer than that at low concentrations of butralin. When applied to environmental bioremediation, pesticide half-life is an essential factor. However, butralin biodegradation has not been studied using a first-order kinetic model. There is an urgent need to explore additional degradation strains to study trends in the biodegradation kinetics of butralin.

Identification of metabolites and analysis of metabolic pathways in butralin-degrading strain FC02

The database used to identify degradation products was sourced from the NIST library database. Table S5 lists the *RT* values of the degradation products, mass-to-charge ratios (m/z), and compound structures. Based on the LC-MS/MS analysis of the metabolites, five metabolite structures of butralin were identified, and then analyzed using MS/MS Fig. 5. Fig. 6 shows the putative metabolic pathway of bacterial butralin degradation. The possible metabolic pathway of strain FC02 to degrade butralin was analyzed as shown in Fig. 6. First, the nitro group of butralin undergoes a reduction reaction, preferentially reducing a nitro group of butralin to form metabolite A and

Table 1 Regression equation and half-life of various initial butralin concentrations[a]

Butralin concentration (mg L^{-1})	Model fitting parameters				
	S_0 (mg L^{-1})	Regression equation	k (d^{-1})	$t_{1/2}$ (d)	R^2
100	100	$C_t = 104.31e^{-0.17732x}$	0.17732	3.90	0.97605
200	200	$C_t = 101.81e^{-0.18294x}$	0.18294	3.79	0.99543
450	450	$C_t = 99.00e^{-0.17539x}$	0.17539	3.95	0.96425
900	900	$C_t = 92.26e^{-0.06685x}$	0.06685	10.37	0.93185
1800	1800	$C_t = 86.51e^{-0.02129x}$	0.02129	32.56	0.68686
3600	3600	$C_t = 95.59e^{-0.01414x}$	0.01414	49.02	0.91475

[a] k indicates the degradation constant (d^{-1}); $t_{1/2}$ indicates the half-life (d); R^2 indicatesthe coefficient of determination.

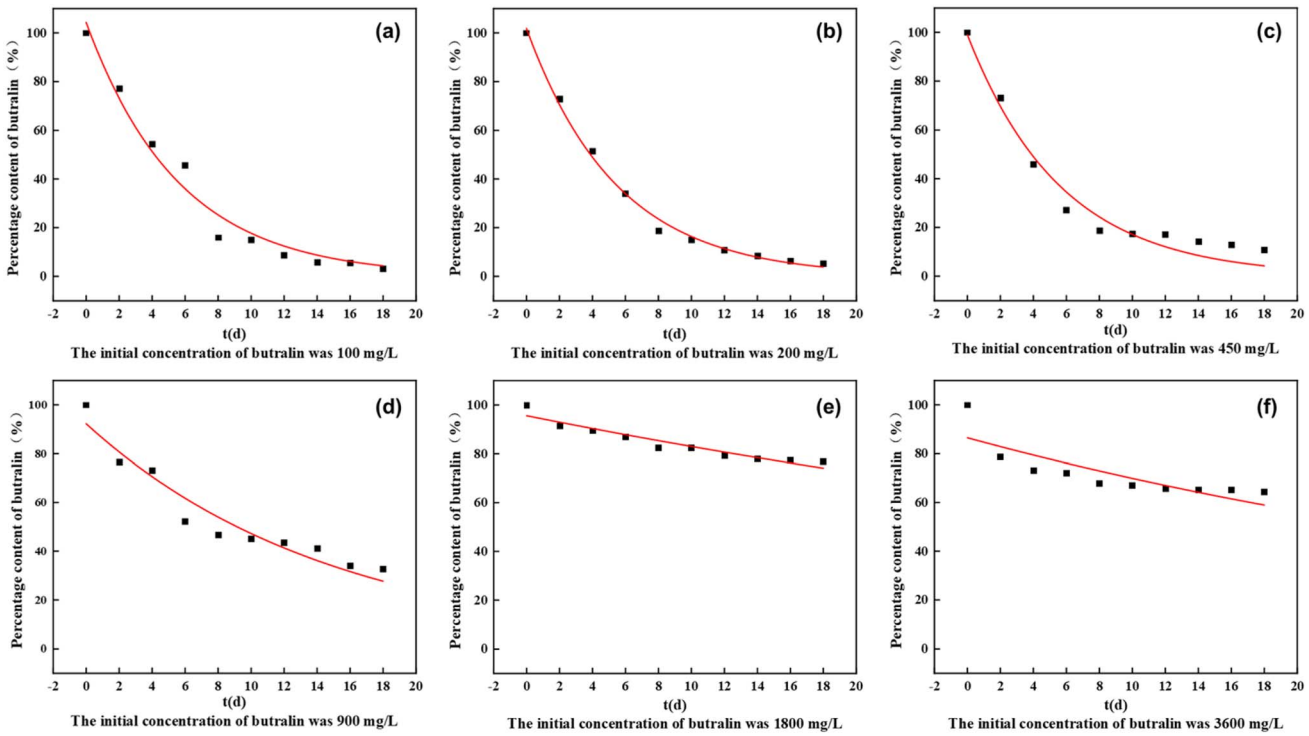

Fig. 4 First-order kinetic model for six different concentrations of butralin. The initial butralin concentrations of were 100 mg L^{-1} (a), 200 mg L^{-1} (b), 450 mg L^{-1} (c), 900 mg L^{-1} (d), 1800 mg L^{-1} (e), 3600 mg L^{-1} (f).

further carrying out C–H Hydroxylation and *N*-dealkylation to form metabolite B. The intermediate product is considered to be carbinolamine, which was not detected because of the rapid reaction. Methanolamine is dealkylated to form metabolite C, methylamine forms metabolite D through hydrolysis, and metabolite D undergoes C–H Hydroxylation and elimination reaction to form metabolite E. This conclusion is the same as that of a previously reported first step in butralin biodegradation.[17,47] Nitrogen reduction is the primary degradation pathway.[49–52] The intermediates 1-{[6-amino-4-(2-methylprop-2-yl)-2-nitrophenyl]amino}ethan-1-ol and 2-{[(2*E*)-but-3-en-2-ylidene]amino}-5-(2-methylprop-2-yl)-3-nitroaniline were identified for the first time. Metabolites B and E are two new metabolites, that show changes compared with the degradation pathway of butralin. When the metabolite structure was determined, a small amount of metabolite F was found, which

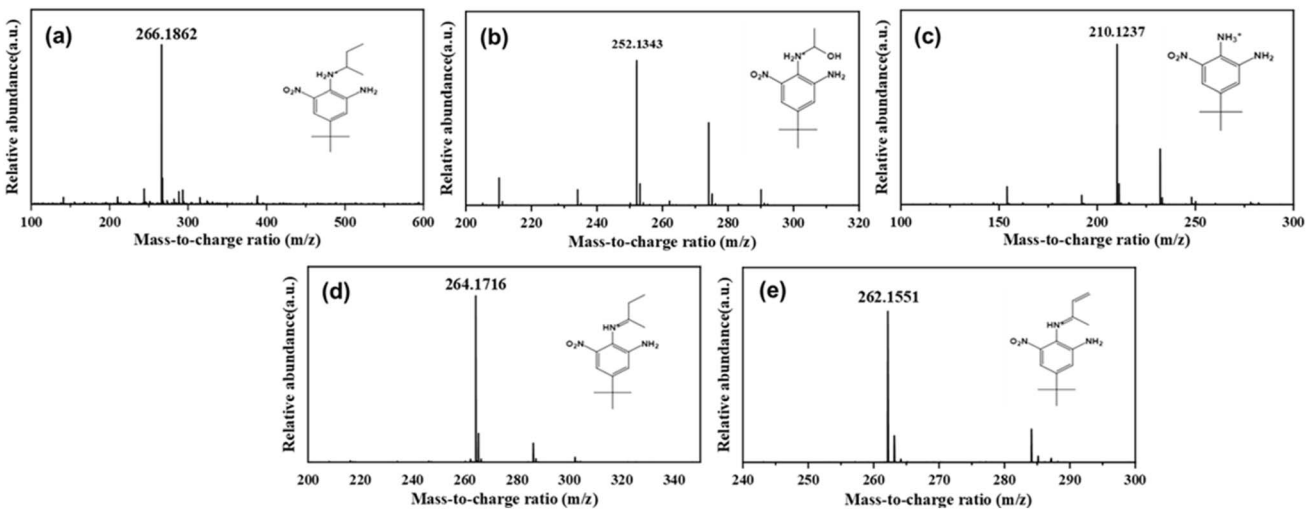

Fig. 5 MS/MS analysis metabolites of butralin degraded by strain FC02. Note: metabolite (a) retention time (RT) = 19.9 min; (b) RT = 13.2 min; (c) RT = 15.0 min; (d) RT = 17.8 min; (e) RT = 14.7 min.

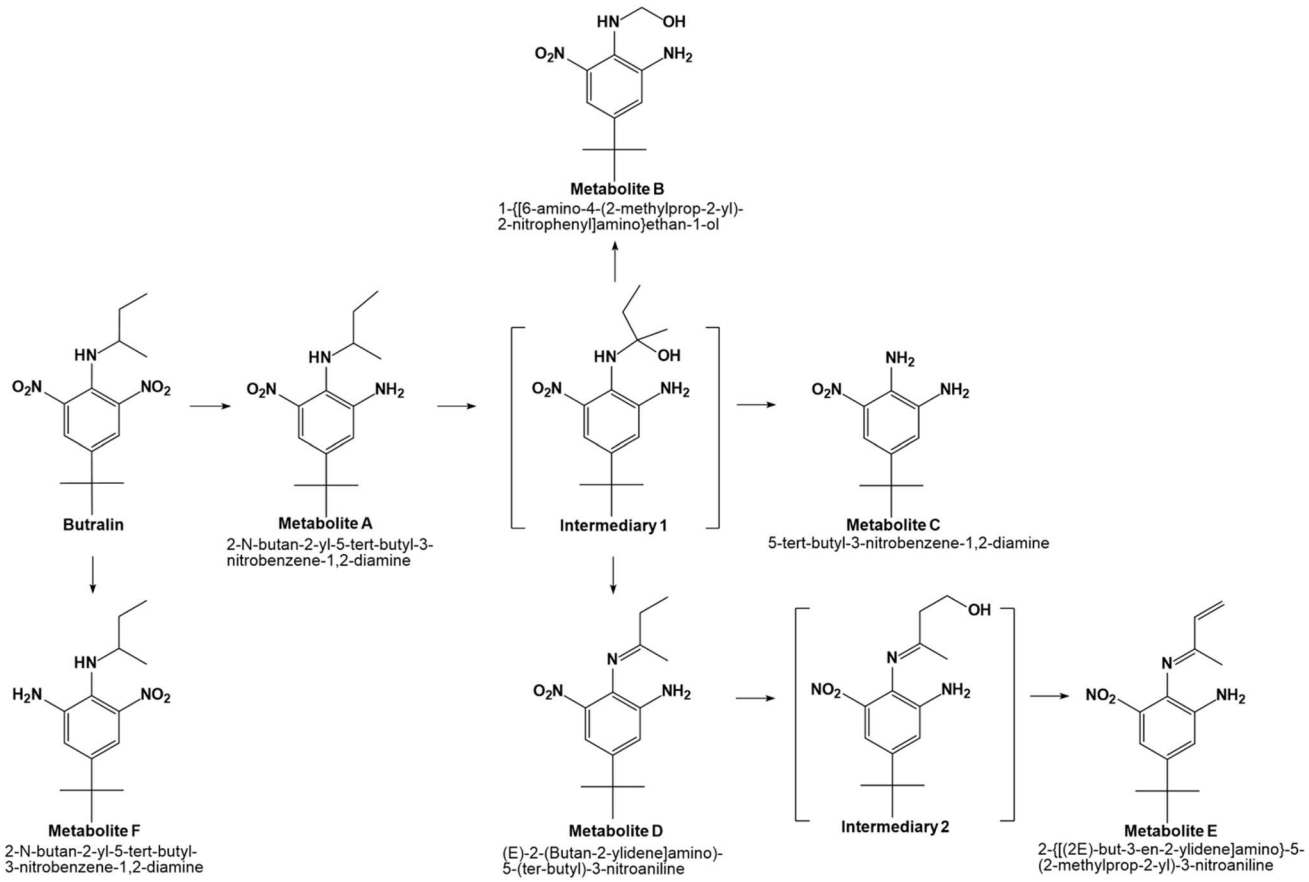

Fig. 6 Putative metabolic pathway for butralin degradation in *Bacillus velezensis*.

was analyzed and confirmed to be an isomer of metabolite A. Therefore, both nitro groups of butralin underwent reduction reactions. Compared with previous studies, strain FC02 employs a more complex degradation pathway, involving synergistic effects of multiple metabolic processes, including nitro-reduction, dealkylation, and hydroxylation. This study confirmed that FC02 degrades butralin through multiple mechanisms, enhancing both environmental adaptability and degradation efficiency.

Analysis of genomic information of butralin degrading strain FC02

The genome of strain FC02 was contained 3 964 910 biological processes (BP), and the average cellular components (CC) content of the high-quality data was 47.44%. Where 3733 protein-coding genes were detected, accounting for 88.65% of the whole genes, as determined using six functional protein annotation databases (Fig. 7). Plasmids, pseudogenes, or gaps were not detected in the FC02 genome. There were 86 tRNAs, 27 rRNAs, 28 housekeeping genes, and other 81 sRNAs in the genome of strain FC02. The GO database annotated the functional protein genes according to BP, CC, and molecular function (MF). A total of 1153 genes were annotated to biological processes, involving most genes related to the regulation of transcription and translation and most minor genes related to tRNA-guanine transglycosylation and recombination repair, each with only one gene. Among the CC, 1138 genes were annotated, most of which were related to integral membrane and plasma membrane components and the, cytoplasm, and only one gene each was related to the septin ring, bacterial-type flagellum, and bacterial nucleoid. A total of 1843 genes in the MF class were annotated, most of which were associated with ATP and DNA binding. In addition, only one gene was related to the recombinase activity.

As shown in Table 2, six potential nitroreductase genes were identified based on annotation of the FC02 genome and the predicted nitroreductases in *Bacillus* screened in the database. Online comparison analysis in the UniProt SwissProt database revealed that amino acid sequences of the six genes were 100% identical to those of nitroreductases homologs of *Bacillus*.[53] Among them, the nitroreductase[54] showed that moderate expression of damaged *B. subtilis bdhA* weakens the pathway of NADH-associated acetoin by-product pools. The nitro moieties of polynitroaromatic compounds can be reduced by single or double-electron mechanisms, represented by two types of bacterial nitroreductases.[55] Type I nitroreductases are responsible for sequentially reducing nitro groups by adding electron pairs from NAD(P)H to yield nitroso, hydroxyamino, and amino derivatives,[56] while Type II enzymes are responsible for single-electron reduction of nitro groups to produce nitroanion

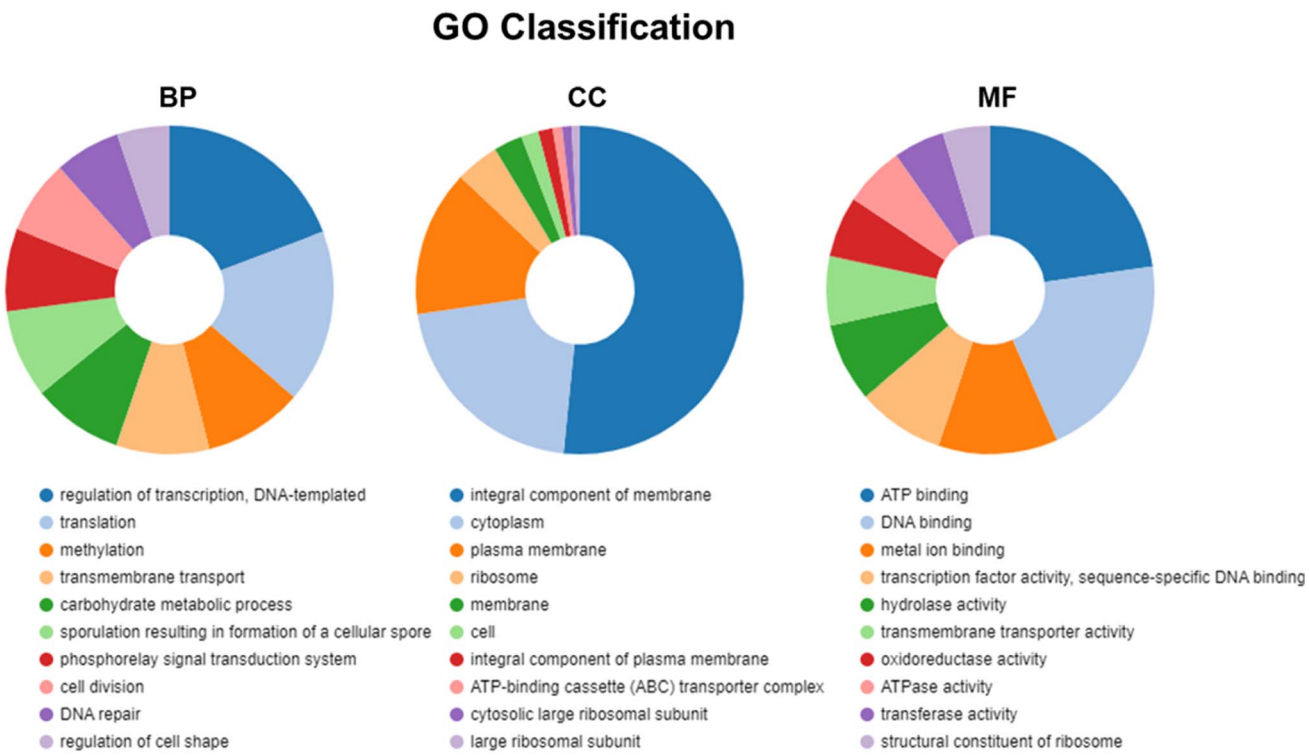

Fig. 7 Gene function annotation of strain FC02 Gene Ontology (GO) functional classification map.

radicals, which continue to oxidize to their original structure while producing superoxide anions.[56,57] Nitroreductases play essential roles in bioremediation.

Transcriptomic analysis

Preliminary analysis of the transcriptome data suggested that there was a total of 3587 expressed genes in the CK group, of which 66 were specifically expressed genes, 3521 were co-expressed genes between the CK and the treatment groups, and 21 genes were specifically expressed genes in the treatment group. The number of co-expressed genes was much higher than that of specifically expressed genes in the CK and treatment groups (Fig. 8a). As shown in Fig. 8b, 1025 genes were differentially expressed in response to butralin, with upregulation of 620 genes (60.49%) and downregulation of 405 genes (39.51%).

GO enrichment analysis in Fig. 8c revealed that 449 genes were differentially expressed, of which 258 were up-regulated, and 191 were down-regulated. In terms of BP, the group treated with butralin was mainly enriched in oxidation-reduction, organonitrogen compound biosynthetic, and amide biosynthetic processes. Relative to CC, the group treated with butralin was primarily enriched in organelles and cell parts. The treat group was mainly enriched in cation and metal ion binding in the MF genes. Transcriptome data showed that the redox process was the most significant in BP. Genes with a nitro reduction function in the first step of the parabutylene metabolic pathway were further analyzed, and six nitroreductase genes of the nitro reductase family were identified: WP-003155509.1, WP-003156324.1, WP-004393039.1, AHZ-15989.1, WP-024084851.1, and WP-003150944.1. After comparing the six nitroreductase genes in the entire genome, WP-003155509.1 and WP-003156324.1 matched. The qRT-PCR results also confirmed the upregulation of these two genes (Fig. 9). WP-003155509.1 and WP-003156324.1 are nitroreductase genes, and we hypothesized that the first step in the degradation of

Table 2 Nitroreductase gene in the genome of strain FC02 and homologous comparison with *Bacillus*

Gene number	Size(bp)	Accession	Amino acid sequence homology	Gene description
gene3808	639	WP-004393039.1	100%	Nitrorreductase family proteins
gene0276	621	WP-024084851.1	100%	Nitrorreductase family proteins
gene3570	750	WP-003150944.1	100%	Oxygen- insensitive NADPH nitroreductase
gene1908	609	AHZ-15989.1	100%	Nitroreductase
gene0394	747	WP-003156324.1	100%	Oxygen- insensitive NADPH nitroreductase
gene0826	585	WP-003155509.1	100%	Nitroreductase

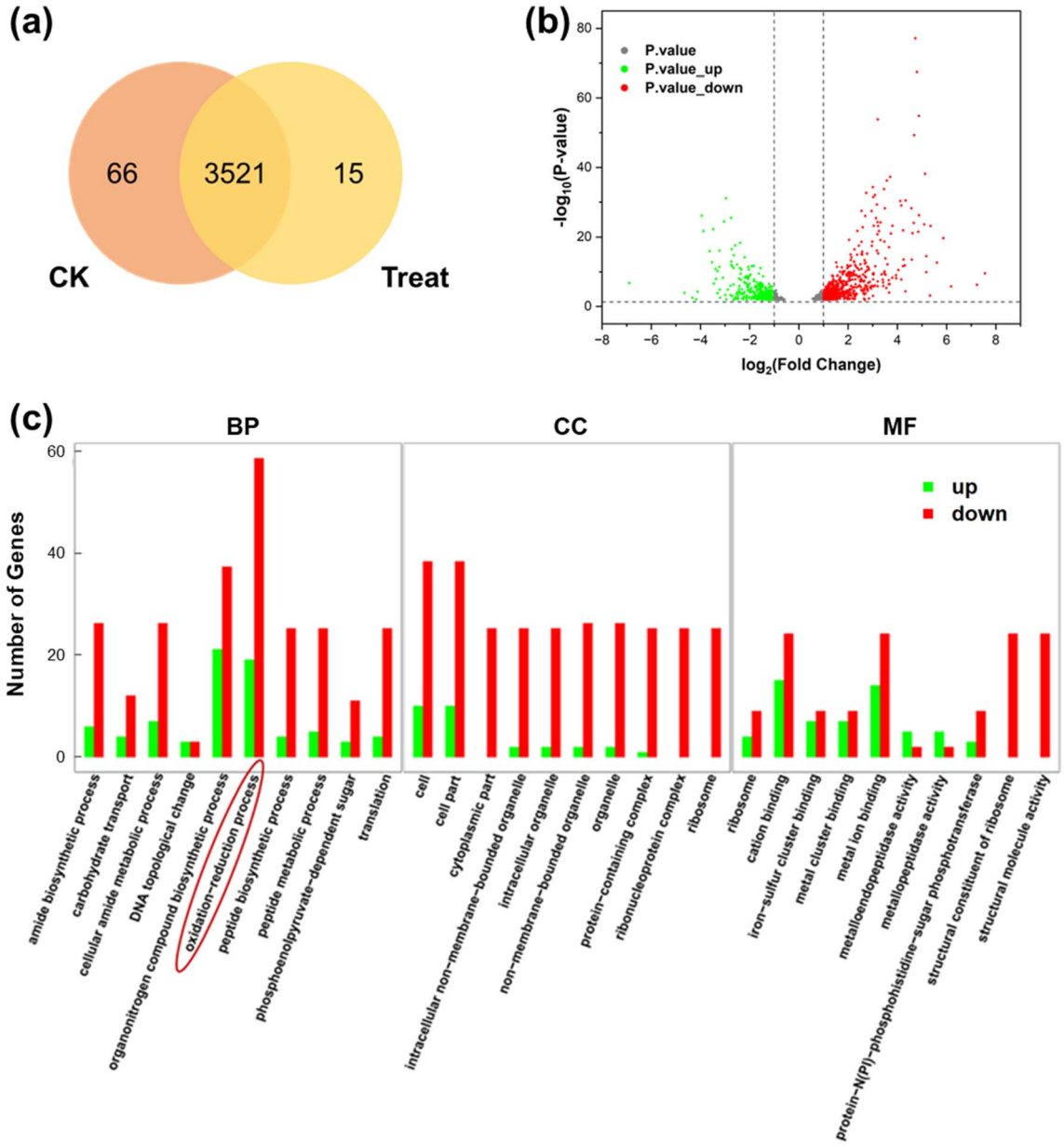

Fig. 8 Analysis of gene expression and its differences including (a) Venn diagram, (b) volcano diagram, and (c) GO enrichment classification column diagram. Biological processes (BP) showing significant enrichment in oxidation-reduction and organonitrogen compound biosynthesis. Cellular components (CC) highlighting predominant localization to membranes and cytoplasmic regions. Molecular functions (MF) demonstrating dominant roles in cation binding and oxidoreductase activities.

para-reductase was completed. Jun Yang[57] purified a nitroreductase from *S. mirabilis* DUT001,[58] which could be used for the nitroreduction of polycyclic nitroaromatic compounds. WP-003155509.1 is an oxygen-insensitive (type I) nitroreductase, belonging to a class of bacterial enzymes that contain FMN and catalyzes the reduction of nitro compounds to produce nitroso, hydroxylamine, or amino derivatives.[55,59] WP-003156324.1 is an NAD(P)H-dependent, oxygen-insensitive type I enzyme. These enzymes can catalyze the oxidation or reduction of CH or CH2 groups, leading to the addition or removal of hydroxyl groups in most cases.[60,61] Therefore, we hypothesized that intermediates 1 and 2 are produced by an NAD(P)H-dependent oxidoreductase.

Degradation effect of strain FC02 on butralin in tobacco leaves

Fig. 10 shows that butralin concentration in Treatment with FC02 decreased from 100 mg kg^{-1} to 0 mg kg^{-1} after 10 days, with the degradation efficiency 100%. The raw chromatograms in SI materials. While the concentration in control treatment decreases from 100 mg kg^{-1} to 67.02 mg kg^{-1} after 10 days. The experimental results shown that the strain FC02 effectively enhanced butralin degradation in tobacco. To facilitate large-scale implementation, future studies should prioritize field trials to systematically evaluate the strain's butralin degradation performance under real agricultural conditions.

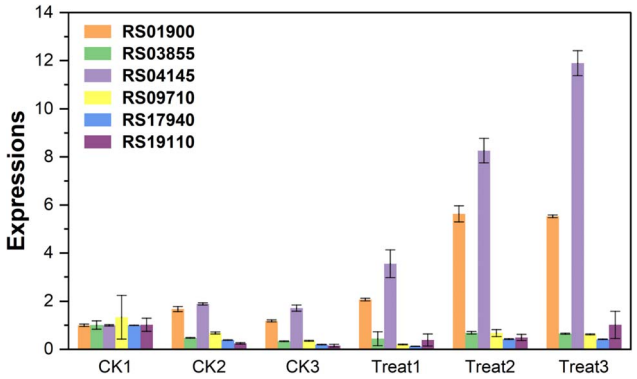

Fig. 9 qRT-PCR validation of nitroreductase genes expression in strain FC02.

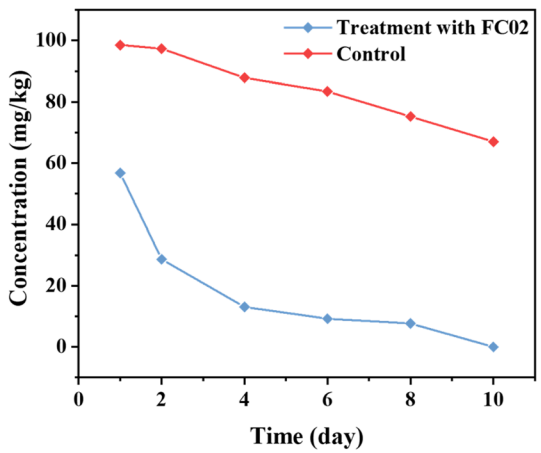

Fig. 10 Degradation curves of FC02 on butralin in tobacco leaves.

Conclusions

In this study, strain FC02 isolated from wooden blocks of the shipwreck "Nanhai I" exhibited a butralin efficient degradation capacity on butralin. The optimal biodegradation conditions were as follows: OD_{600} = 2.5, 35.0 °C, and initial pH 8.4. The optimal conditions for butralin degradation by this strain have been reported for the first time. Based on this our results, biodegradation pathways for butralin have been proposed, including nitro reduction, hydroxylation, dealkylation, and elimination. Six nitroreductase genes were analyzed at both genome-wide and transcriptome levels, and the high expression of two nitroreductase genes were determined. The highly efficient butralin-degrading strain FC02 found in this study has broad prospects for biodegradation, which provides the support for its future application in butralin remediation.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request. Supplementary information: provide the levels of three test factors in butralin degradation optimization with strain FC02, Box–Behnken test design and regression analysis results. As well as anova of quadratic regression equation of strain FC02, chromatographic properties of metabolites of butralin by strain FC02. In addition to HPLC method, chromatographic conditions, and raw chromatograms of butralin in tobacco leave. See DOI: https://doi.org/10.1039/d5ra02138c.

References

1 Q. P. Wang, J. H. Li, R. Y. Li, X. L. Pan, F. S. Dong and X. M. Wu, Rapid elimination of butralin residues on tobacco, in water and soils by newly isolated *Bacillus* sp. LY05, *J. Environ. Chem. Eng.*, 2023, **11**(1), 109213.
2 X. X. Xu, X. Guo, S. S. Song, A. H. Wu, C. L. Xu, H. Kuang and L. Q. Liu, Gold-based strip sensor for the rapid and sensitive detection of butralin in tomatoes and peppers, *Food Addit. Contam.,:Part A*, 2022, **39**(7), 1255–1264.
3 C. D. Li, R. Liu, L. Li, W. Li, Y. J. He and L. F. Yuan, Dissipation behavior and risk assessment of butralin in soybean and soil under field conditions, *Environ. Monit. Assess.*, 2017, **189**(9), 476.
4 L. H. Yang, X. X. Song, X. G. Zhou, Y. Z. Zhou, D. X. Gong, H. F. Luo, Y. C. Deng, D. X. Yang and L. Chen, Residual behavior and risk assessment of butralin in peanut fields, *Environ. Monit. Assess.*, 2019, **192**(1), 62.
5 M. C. Van and L. R. Fisher, Azoxystrobin, butralin, and flumetralin residues in flue-cured tobacco, *Tob. Sci.*, 2014, **51**, 23–28.
6 W. J. Huang, R. Kong, L. J. Chen and Y. X. An, Physiological responses and antibiotic-degradation capacity of duckweed (*Lemna aequinoctialis*) exposed to streptomycin, *Front. Plant Sci.*, 2022, **13**, 1065199.
7 A. Giglio and M. L. Vommaro, Dinitroaniline herbicides: a comprehensive review of toxicity and side effects on animal non-target organisms, *Environ. Sci. Pollut. Res.*, 2022, **29**(51), 76687–76711.
8 A. A. Refaie, A. Ramadan, N. M. Sabry, W. K. B. Khalil and A. T. H. Mossa, Over-gene expression in the apoptotic, oxidative damage and liver injure in female rats exposed to butralin, *Environ. Sci. Pollut. Res.*, 2020, **27**(25), 31383–31393.
9 Q. G. Liao, Y. M. Zhou, L. G. Luo, L. B. Wang and X. H. Feng, Determination of twelve herbicides in tobacco by a combination of solid-liquid-solid dispersive extraction using multi-walled carbon nanotubes, dispersive liquid-liquid micro-extraction, and detection by GC with triple quadrupole mass spectrometry, *Microchim. Acta*, 2014, **181**(1–2), 163–169.
10 C. Zhang, B. Y. Jin, J. Q. Liu, Q. P. Wang and B. Tian, Biodegradation of dinitroaniline herbicides: A

comprehensive review, *Environ. Technol. Innovation*, 2025, **39**, 104225.

11 A. Aswathi, A. Pandey and R. K. Sukumaran, Rapid degradation of the organophosphate pesticide-Chlorpyrifos by a novel strain of Pseudomonas nitroreducens AR-3, *Bioresour. Technol.*, 2019, 292.

12 X. Y. Cai, M. Xu, Y. X. Zhu, Y. Shi and H. W. Wang, Removal of Dinotefuran, Thiacloprid, and Imidaclothiz Neonicotinoids in Water Using a Novel Pseudomonas monteilii FC02–Duckweed (*Lemna aequinoctialis*) Partnership, *Front. Microbiol.*, 2022, **13**, 906026.

13 Y. j. Han, Z. G. Tang, H. F. Bao, D. M. Wu, X. L. Deng, G. W. Guo, B. C. Ye and B. Dai, Degradation of pendimethalin by the yeast YC2 and determination of its two main metabolites, *RSC Adv.*, 2019, **9**(1), 491–497.

14 R. Rani, V. Kumar, Z. Usmani, P. Gupta and A. Chandra, Influence of plant growth promoting rhizobacterial strains *Paenibacillus sp.* IITISM08, *Bacillus sp.* PRB77 and *Bacillus sp.* PRB101 using Helianthus annuus on degradation of endosulfan from contaminated soil, *Chemosphere*, 2019, **225**, 479–489.

15 Q. P. Wang, C. Zhang, B. Tian, L. Han, D. D. Liu, G. Li, S. H. Gui, S. Guy, X. S. Chen and X. M. Wu, Consortia of *Bacillus* sp. LY05 and *Bacillus cereus* LGY06 immobilized on coconut shell charcoal remediates pendimethalin and cadmium contaminated sites in-situ and alleviates peanut's continuous cropping obstacles, *J. Hazard. Mater.*, 2025, **492**, 138168.

16 H. Y. Ni, L. Yao, N. Li, Q. Cao, C. Dai, J. Zhang, Q. He and J. He, Biodegradation of pendimethalin by *Bacillus subtilis* Y3, *J. Environ. Sci.*, 2016, **41**, 121–127.

17 S. Ghatge, Y. R. Yang, S. Moon, W. Y. Song, T. Y. Kim, K. H. Liu and H. G. Hur, A novel pathway for initial biotransformation of dinitroaniline herbicide butralin from a newly isolated bacterium *Sphingopyxis sp.* strain HMH, *J. Hazard. Mater.*, 2021, 402.

18 R. Y. Stanier, N. J. Palleroni and M. Doudoroff, The aerobic pseudomonads: a taxonomic study, *J. Gen. Microbiol.*, 1966, **43**(2), 159–271.

19 S. H. Chen, Y. H. Dong, C. Q. Chang, Y. Y. Deng, X. F. Zhang, G. H. Zhong, H. W. Song, M. Y. Hu and L. H. Zhang, Characterization of a novel cyfluthrin-degrading bacterial strain *Brevibacterium aureum* and its biochemical degradation pathway, *Bioresour. Technol.*, 2013, **132**, 16–23.

20 D. R. Boone, R. W. Castenholz and G. M. Garrity, *Bergey's Manual® of Systematic Bacteriology*, Springer Science & Business Media, 2001.

21 P. Bhatt, Y. H. Huang, E. R. Rene, A. J. Kumar and S. H. Chen, Mechanism of allethrin biodegradation by a newly isolated *Sphingomonas trueperi* strain CW3 from wastewater sludge, *Bioresour. Technol.*, 2020, 305.

22 Z. Q. Lin, S. M. Pang, Z. Zhou, X. Z. Wu, J. Y. Li, Y. H. Huang, W. P. Zhang, Q. Q. Lei, P. Bhatt, S. Mishra and S. H. Chen, Novel pathway of acephate degradation by the microbial consortium ZQ01 and its potential for environmental bioremediation, *J. Hazard. Mater.*, 2022, **426**, 127841.

23 S. S. Patil and H. M. Jena, Statistical Optimization of Phenol Degradation by *Bacillus pumilus* OS1 Using Plackett-Burman Design and Response Surface Methodology, *Arabian J. Sci. Eng.*, 2015, **40**(8), 2141–2151.

24 B. Thangagiri, A. Sakthivel, K. Jeyasubramanian, S. Seenivasan and J. D. Raja, Modeling and optimization of removal of Pb (II) in aqueous solutions by biochar derived from neem leaves using central composite design of response surface methodology, *Biomass Convers. Biorefin.*, 2023, **14**, 16019–16034.

25 Q. P. Wang, J. H. Li, R. Y. Li, X. L. Pan, F. S. Dong and X. M. Wu, Rapid elimination of butralin residues on tobacco, in water and soils by newly isolated *Bacillus sp.* LY05, *J. Environ. Chem. Eng.*, 2023, **11**(1), 109213.

26 M. Cycoń, A. Mrozik and Z. Piotrowska-Seget, Bioaugmentation as a strategy for the remediation of pesticide-polluted soil: a review, *Chemosphere*, 2017, **172**, 52–71.

27 S. Terzic, N. Udikovic-Kolic, T. Jurina, I. Krizman-Matasic, I. Senta, I. Mihaljevic, J. Loncar, T. Smital and M. Ahel, Biotransformation of macrolide antibiotics using enriched activated sludge culture: Kinetics, transformation routes and ecotoxicological evaluation, *J. Hazard. Mater.*, 2018, **349**, 143–152.

28 S. Kou, L. Q. Liu, S. S. Song, H. Kuang, C. L. Xu and X. L. Wu, Development of a sensitive lateral flow immunoassay for the rapid detection of butralin in fruits, *Food Control*, 2024, **162**, 110402.

29 Z. W. Tan, J. C. Chen, Y. L. Liu, L. Chen, Y. Q. Xu, Y. X. Zou, Y. T. Li and B. N. Gong, The survival and removal mechanism of *Sphingobacterium changzhouense* TC931 under tetracycline stress and its' ecological safety after application, *Bioresour. Technol.*, 2021, **333**, 125067.

30 K. J. Duelge, U. Nishshanka and H. G. De Alwis, An LC-MS/MS method for the determination of antibiotic residues in distillers grains, *J. Chromatogr. B:Anal. Technol. Biomed. Life Sci.*, 2017, **1053**, 81–86.

31 S. Kim, S. Park, E. Park, J. Kim, S. Ghatge, H. Hur and S. Rhee, Structure and substrate specificity determinants of NfnB, a dinitroaniline herbicide–catabolizing nitroreductase from *Sphingopyxis* sp. strain HMH, *J. Biol. Chem.*, 2021, **297**(4), 101143.

32 R. M. Bi, M. H. Ou, S. R. Zhou, S. C. Geng, Y. X. Zheng, J. H. Chen, R. J. Mo, Y. Li, G. Z. Xiao, X. Y. Chen, S. Y. Zhai, A. H. Zhang and B. S. Fang, Degradation strategies of pesticide residue: from chemicals to synthetic biology, *Synth. Syst. Biotechnol.*, 2023, **8**(2), 302–313.

33 S. Bose, P. S. Kumar, D.-V. N. Vo, N. Rajamohan and R. Saravanan, Microbial degradation of recalcitrant pesticides: a review, *Environ. Chem. Lett.*, 2021, **19**(4), 3209–3228.

34 S. Rana, P. Mardarveran, R. Gupta, L. Singh and Z. ab Wahid, Role of Microbes in Degradation of Chemical Pesticides. in *Microbes and Enzymes in Soil Health and Bioremediation*, ed. A. Kumar and S. Sharma, Springer, Singapore, 2019, pp. 255–275.

35 J. Y. Zhao, Y. D. Jiang, L. M. Gong, X. F. Chen, Q. L. Xie, Y. Jin, J. Du, S. F. Wang and G. Liu, Mechanism of β-cypermethrin

metabolism by *Bacillus cereus* GW-01, *Chem. Eng. J.*, 2022, **430**, 132961.

36 B. Zeng, Y. T. Liu, Z. R. Feng, F. R. Chen, S. F. Wu, C. Bass and C. F. Gao, The overexpression of cytochrome P450 genes confers buprofezin resistance in the brown planthopper, *Nilaparvata lugens* (Stål), *Pest Manage. Sci.*, 2023, **79**(1), 125–133.

37 Z. X. Cao, M. M. Wang, T. Zhou, A. Xu and H. Du, Whole-Genome Sequencing Reveals Germ Cell Mutagenicity of α-Endosulfan in *Caenorhabditis elegans*, *Environ. Sci. Technol.*, 2022, **56**(22), 16024–16032.

38 X. D. Chu, M. K. Awasthi, Y. Y. N. Liu, Q. H. Cheng, J. B. Qu and Y. Sun, Studies on the degradation of corn straw by combined bacterial cultures, *Bioresour. Technol.*, 2021, **320**, 124174.

39 W. Z. Wu, W. J. Chen, Z. Q. Lin, Y. H. Huang, T. N-M El Sebai, N. Alansary, D. E. El-Hefny, S. Mishra, P. Bhatt, H. X. Lü and S. H. Chen, Rapid Biodegradation of the Organophosphorus Insecticide Acephate by a Novel Strain *Burkholderia* sp. A11 and Its Impact on the Structure of the Indigenous Microbial Community, *J. Agric. Food Chem.*, 2023, **71**, 5261–5274.

40 R. Long, W. J. Yang and G. L. Huang, Optimization of fermentation conditions for the production of epothilone B, *Chem. Biol. Drug Des.*, 2020, **96**(2), 768–772.

41 A. Shilli, S. K. Manikandan and V. Nair, Application of Box-Behnken Design in Optimization of the Okra (*Abelmoschus esculentus* L.) Plant Growth in Loamy Sand Soil, *J. Soil Sci. Plant Nutr.*, 2023, **23**, 2625–2636.

42 K. N. Kragh, M. Alhede, M. Rybtke, M. Rybtke, C. Stavnsberg, P. Ø. Jensen, T. Tolker-Nielsen, M. Whiteley and T. Bjarnsholt, The Inoculation Method Could Impact the Outcome of Microbiological Experiments, *Appl. Environ. Microbiol.*, 2018, **84**(5), e02264.

43 J. Yu, J. Gu, X. J. Wang, L. S. Lei, H. H. Guo, Z. L. Song and W. Sun, Exploring the mechanism associated with methane emissions during composting: Inoculation with lignocellulose-degrading microorganisms, *J. Environ. Manage.*, 2023, 325.

44 W. G. Birolli, R. N. Lima and A. L. M. Porto, Applications of Marine-Derived Microorganisms and Their Enzymes in Biocatalysis and Biotransformation, the Underexplored Potentials, *Front. Microbiol.*, 2019, **10**, 1453.

45 S. H. Chen, J. J. Luo, M. Y. Hu, P. Geng and Y. B. Zhang, Microbial detoxification of bifenthrin by a novel yeast and its potential for contaminated soils treatment, *PLoS One*, 2012, **7**(2), e30862.

46 Y. Zhai, K. Li, J. L. Song, Y. H. Shi and Y. C. Yan, Molecular cloning, purification and biochemical characterization of a novel pyrethroid-hydrolyzing carboxylesterase gene from *Ochrobactrum anthropi* YZ-1, *J. Hazard. Mater.*, 2012, **221**, 206–212.

47 H. Y. Ni, N. Li, J. G. Qiu, Q. Chen and J. He, Biodegradation of Pendimethalin by *Paracoccus* sp P13, *Curr. Microbiol.*, 2018, **75**(8), 1077–1083.

48 N. Trivedi and A. Dubey, Degradation studies of pendimethalin by indigenous soil bacterium *Pseudomonas* strain PD1 using spectrophotometric scanning and FTIR, *Arch. Microbiol.*, 2021, **203**(7), 4499–4507.

49 C. A. Aswathy, P. Nagaraaj, K. Kalanidhi and S. G. Vanthana, Bio-based cellulose supported copper oxide nanoparticles for the reduction of nitro-aromatic compounds, *Inorg. Chim. Acta*, 2023, **545**, 121243.

50 S. H. Kim, S. Park, E. Park, J. H. Kim, S. Ghatge, H. G. Hur and S. Rhee, Structure and substrate specificity determinants of NfnB, a dinitroaniline herbicide–catabolizing nitroreductase from *Sphingopyxis* sp. strain HMH, *J. Biol. Chem.*, 2021, **297**(4), 101143.

51 H. Y. Ni, F. Wang, N. Li, L. Yao, C. Dai, Q. He, J. He and Q. Hong, Pendimethalin Nitroreductase Is Responsible for the Initial Pendimethalin Degradation Step in *Bacillus subtilis* Y3, *Appl. Environ. Microbiol.*, 2016, **82**(24), 7052–7062.

52 C. C. Zhu, H. N. Huang and Y. G. Chen, Recent advances in biological removal of nitroaromatics from wastewater, *Environ. Pollut.*, 2022, **307**, 119570.

53 R. Borriss, A. Danchin, C. R. Harwood, C. Médigue, E. P. C. Rocha, A. Sekowska and D. Vallenet, *Bacillus subtilis*, the model Gram-positive bacterium: 20 years of annotation refinement, *Microb. Biotechnol.*, 2018, **11**(1), 3–17.

54 X. Zhang, R. Z. Zhang, T. Bao, Z. M. Rao, T. W. Yang, M. J. Xu, Z. H. Xu, H. Z. Li and S. T. Yang, The rebalanced pathway significantly enhances acetoin production by disruption of acetoin reductase gene and moderate-expression of a new water-forming NADH oxidase in Bacillus subtilis, *Metab. Eng.*, 2014, **23**, 34–41.

55 M. Dolores Roldan, E. Perez-Reinado, F. Castillo and C. Moreno- Vivián, Reduction of polynitroaromatic compounds: the bacterial nitroreductases, *FEMS Microbiol. Rev.*, 2008, **32**(3), 474–500.

56 F. J. Peterson, R. P. Mason, J. Hovsepian and J. L. Holtzman, Oxygen-sensitive and -insensitive nitroreduction by *Escherichia coli* and rat hepatic microsomes, *J. Biol. Chem.*, 1979, **254**(10), 4009–4014.

57 R. P. Mason and J. L. Holtzman, The role of catalytic superoxide formation in the O2 inhibition of nitroreductase, *Biochem. Biophys. Res. Commun.*, 1975, **67**(4), 1267–1274.

58 J. Yang, B. Xie, J. Bai and Q. Yang, Purification and characterization of a nitroreductase from the soil bacterium *Streptomyces mirabilis*, *Process Biochem.*, 2012, **47**(5), 720–724.

59 A. Schenzle, H. Lenke, J. C. Spain and H. J. Knackmuss, Chemoselective nitro group reduction and reductive dechlorination initiate degradation of 2-chloro-5-nitrophenol by *Ralstonia eutropha* JMP134, *Appl. Environ. Microbiol.*, 1999, **65**(6), 2317–2323.

60 M. Romig, M. Eberwein, D. Deobald and A. Schmid, Reactivation and long-term stabilization of the [NiFe] Hox hydrogenase of *Synechocystis* sp. PCC6803 by glutathione after oxygen exposure, *J. Biol. Chem.*, 2025, **301**(1), 108086.

61 L. S. Vidal, C. L. Kelly, P. M. Mordaka and J. T. Heap, Review of NAD(P)H-dependent oxidoreductases: Properties, engineering and application, *Biochim. Biophys. Acta, Proteins Proteomics*, 2018, **1866**(2), 327–347.