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Isolation, identification and subsequent application of Gram-positive bacterial strains for the bioremediation of benzophenone

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Benzophenone (BZP) is a persistent environmental pollutant that can induce serious negative effects on the human health and aquatic life. Because of its hydrophobic nature and resistance to natural degradation, it tends to bioaccumulate in the aquatic ecosystem. The present study was aimed at developing an efficient bioremediation system for the degradation of BZP in wastewater. A total of 150 bacterial strains were isolated from wastewater collected from the twin cities of Rawalpindi and Islamabad, Pakistan. Approximately 86% of the isolates were identified in samples of municipal wastewater, and 14% were isolated in the effluent of soap industries. The potential of the isolated strains was assessed for the degradation of BZP. Based on the initial screening, only 3.3% of the isolates were able to produce biomass at different concentrations (10–1000 mg L^{-1}) after 48 h of incubation. Following the first screening, the two most effective bacterial isolates, *Bacillus cereus* (DK2) and *Bacillus pumilus* (S4), identified through their cell growth and morphological, physiological, and phylogenetic characteristics, were selected for further investigation. In the first 72 h, DK2 and S4 degraded more than 60% of BZP. DK2 showed 67.5% degradation, while S4 showed 69.5% degradation. The consortium (DK2 and S4) showed 86% degradation after 72 hours of incubation in liquid MSM under aerobic conditions. Degradation behaviour was accompanied by growth (CFU mL^{-1}) after 120 hours. The BZP degradation reached 100% ± 2.35% after 96 h under the optimum environmental conditions, which included a pH of 7, an incubation temperature of 30 °C, 1.0 g L^{-1} of ammonium nitrate as the nitrogen source, and yeast extract (2.0 g L^{-1}) as an additional carbon source. Gas chromatography-mass spectrometer (GC-MS) analysis exhibited a multistage degradation pathway that included benzophenone ring cleavage and producing compounds like phenyl cyclohexyl ketone, benzophenone dimethyl ketal, 2-cyclohexen-1-one, hydroxylation, demethylation, benzcarbothioc acid, and heptacosane. These compounds are less harmful substances than parent compound. The degradation data was best-fitted with the pseudo-first-order (PFO1) kinetic model, as evident from the rate constant of 0.8 for S4, and pseudo-second-order model (PSO2) for DK2, as indicated by the rate constant of 0.9. The present work provides insights into the biological transformation of BZP and highlights that *Bacillus cereus* and *Bacillus pumilus*, both individually and in a mixed consortium, are promising strains for the bioremediation of wastewater contaminated with BZP.

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Introduction

Aquatic ecosystems are frequently contaminated by a variety of contaminants, such as xenobiotics, carcinogens, and endocrine disruptors, which are generated by manufacturing industries, commercial settlements, municipalities, and agricultural operations.[1–3] Worldwide, the production of municipal wastewater is estimated to be 360 billion m^3 per year.[4] This wastewater is mostly discharged into surface water, often with minimal or no treatment, particularly in developing nations. Chemicals such as benzophenone (diphenyl ketone, $C_{13}H_{10}O$, BZP) are regarded as hazardous pollutants in the environment because of their widespread use and severe environmental concerns.[5] These substances

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are widely used in personal care products including soap as color protectors, perfumes as fragrance enhancers, and UV filters to prevent UV rays from penetrating the skin.[6,7] These chemicals ultimately find their way into the aquatic environment due to their hydrophilic character and incomplete removal at wastewater treatment plants (WWTPs) and also due to the discharge of untreated municipal wastewater. Their presence is frequently reported in the environment, including surface water, groundwater, and sediments.[8,9] The levels of BZPs in the river water of Europe vary from 233 ng L^{-1} to 395 ng L^{-1}.[10] An estimated 10 000 tonnes of UV filters, a single category of personal care products, are produced worldwide yearly.[11,12] It is anticipated that the market for personal care products would grow from $552.60 billion in 2022 to $750.53 billion in 2032, which will eventually lead to an increase in the concentration of BZP in environmental compartments.[13]

BZPs have the potential to harm ecosystems and human health if they are released into the environment without converting them into less hazardous form. The toxicity of BZPs to aquatic animals, algae, and coral reefs has been documented in a number of investigations.[14,15] Moreover, research revealed that BZP and its derivatives are frequently utilized benzophenone-based UV filters, which are identical in their fundamental structure and only vary in their substituents, and can build up in fish tissue, with the gut showing the greatest accumulation, followed by the brain and liver.[16] Higher doses (30 μg L^{-1} and 300 μg L^{-1}) of BZP reduced acetylcholinesterase activity, which functions to convert the important neurotransmitter acetylcholine into acetic acid along with choline.[16] This dissociation stops nerve cells from communicating with their target, which reduces their function. Acetylcholine stimulates muscle contraction and promotes movement in the human body when it is present in the neuromuscular junction. It repressed cortisol release from the brain, whereas exposure to 1.0 μg L^{-1} of this compound increased the secretion of cortisol in the brain. An important hormone that impacts almost all the tissues and organs in the body is cortisol (manages the stress response in the body). BZP exposure altered apoptosis (a type of controlled cell death), hepatic metabolism (metabolism of amino acids and proteins), inflammatory immunity (immune system reaction to injury or illness is inflammation), and oxidative stress in the liver (structural and functional abnormalities in the liver), but they also interfered with these activities in the gut (digestion of food).[16] BZPs have also been reported to have adverse effects on the human liver.[17]

The water solubility of BZPs (at 25 °C) is 37 mg L^{-1} and their octanol/water partition coefficient, $\log P_{ow}$, is 3.38, indicating their high lipophilicity and low solubility in water, and they decompose when the temperature increases above 320 °C. BZP is classified as an aromatic compound, which contains two phenyl (benzene) rings. Its water solubility of 37 mg L^{-1} is considered low, which is due to its organic nature, as organic compounds are considered less water soluble compared to inorganic compounds.[74] It resists natural biodegradation processes and persists in the environment, where it bioaccumulates in the fatty tissues of aquatic organisms and biomagnifies within the food chain and food webs in the ecosystem.[18] The toxic effect of BZP can be attributed to its potential inhibitory effects on eukaryotic ribosome biosynthesis and DNA replication. Ultimately, it can be a serious threat to carbon fixation (photosynthesis process), the nitrogen cycle (flow of nitrogen in the environment in a variety of forms), oxygen production (plants and algae produce oxygen), primary productivity (the rate of biomass production by plants per unit area), and photosynthesis by diatoms (unicellular algae) in coastal waters across the globe.[19] Thus, municipal wastewater containing BZPs must be treated given that it is continuously discharged into the environment, endangering human health and the aquatic biota. This is demonstrated by the short- and long-term effects of BZPs on aquatic species and human health.

The development of BZP degradation technologies that mimic natural systems is imperative considering the harmful effects of BZPs and the limitations of current technologies. Bacteria-based bioremediation is considered to be cost effective, environmentally friendly, and analogous to natural treatment systems and can be employed to degrade a range of organic contaminants, including BZPs from wastewater.[20–22] Only three bacterial species have been found to be capable of degrading BZP-3 (chemical formula $C_{14}H_{12}O_3$, a derivative of BZP with a basic structure similar to BZP (to date: *Methylophilus* sp. strain FP-6,[23] *Rhodococcus* sp. USK10,[24] and *Sphingomonas wittichii* strain BP14P.[25] However, despite the fact that some *Bacillus* strains have been proven to be capable of decolorizing dyes,[26] a comparative study of two distinct *Bacillus* strains that can degrade BZP has not been carried out to date. Additionally, only a few studies on the isolation of potential *Bacillus* strains from municipal wastewater and their application for the biodegradation of BZP from municipal sources have been carried out. Moreover, it has been noted that the bacterial degradation of BZP and its derivatives is not as effective as their fungal degradation, highlighting the need for the isolation, identification, and subsequent application of bacteria for the effective degradation of BZP.[27] Furthermore, the elimination or mineralization of organic pollutants is significantly influenced by the physical and chemical properties of the real environmental compartment in which the target pollutant is located;[28] nevertheless, these aspects have not received enough attention.

In the current investigation, the potential of bacteria isolated from municipal wastewater to biodegrade BZP was investigated. A total of 150 bacterial cultures were isolated from wastewater samples that were collected from various locations in the twin cities of Rawalpindi and Islamabad, Pakistan. After screening, only five of the 150 isolates showed tolerance based on the increase in cell biomass at each time period (O.D value = 0.5) in BZP-enriched medium and biodegradation potential of BZP. Finally, *Bacillus cereus* (DK2) and *Bacillus pumilus* (S4), the two most effective bacterial species, were identified *via* 16S rRNA sequencing and used in the optimization and kinetic studies. The selected strains and optimized conditions would provide baseline data for the development of biotreatment systems for BZP-contaminated wastewater on a larger scale.

Materials and methods

Chemicals and culture medium

Isolation of bacteria from municipal wastewater was performed using mineral salt medium (MSM) consisting of sodium chloride

(1.0 g), calcium chloride (0.1 g), magnesium sulfate (0.5 g), potassium dihydrogen phosphate (1.0 g), and disodium hydrogen phosphate (1.0 g) per litre of medium. Benzophenone (99%), phenanthrene-d10, methanol (CH_3OH, 99%), and ethyl acetate (99%) were purchased from Sigma Aldrich. Phenanthrene d10 was used as an internal standard. The stock solution of benzophenone was prepared by dissolving 0.5 g L^{-1} benzophenone in methanol. All chemicals were of analytical grade and used as received.

Wastewater and sludge sampling and analysis

About 25 wastewater and sludge samples were collected from different locations of the twin cities (Rawalpindi, Islamabad-Pakistan). Seven samples were taken from the outlet of different soap industries in Islamabad, and the remaining 18 were collected from residential areas of Rawalpindi, Pakistan as municipal wastewater. According to the guidelines published by the American Public Health Association,[29] the samples were collected and transported using pre-sterilized bottles and Ziplock plastic bags. Fig. S1 displays the sampling locations. Three samples were taken from Nulla Lai (NL), five from Noor Colony (NC), five from Sohan (S), and five from Dhoke Kala Khan (DK) out of the eighteen samples that were collected from Rawalpindi. Table S1 provides the full description of the wastewater characteristics and the sample coordinates.

The wastewater was analysed for its physicochemical characteristics such as pH, total dissolved solids (TDS), electrical conductivity (EC), and chemical oxygen demand (COD). The gravimetric method was used for TDS analysis. The samples were filtered and water was evaporated at 180 °C in a China dish and their residue mass was weighed using an analytical grade balance.[30] The electrical conductivity (EC) and pH and TDS of the wastewater was measured using a digital multimeter (Crison MM + 40). Measuring range: pH = 0.00 to 14.00, conductivity = 0.01 μS cm^{-1} to 200 μS cm^{-1} and TDS = 0.01 mg L^{-1} to 199.9 mg L^{-1}. The chemical oxygen demand (COD) was determined using the open reflux method.[31]

All instruments were standardized with known standards before taking measurements. pH calibration was performed with 1, 2 or 3 buffers chosen from values: pH 2.00, 4.01, 7.00, 9.00 and 10.90 at 25 °C. Conductivity calibration was performed with 1, 2 or 3 standards chosen from values: 147 μS cm^{-1}, 1413 μS cm^{-1}, 12 mS cm^{-1}, at 25 °C. TDS conversion factor: values between 0.40 and 1.00 were adjusted. All experiments were performed in three replicates and the data presented is the mean of three replications.

Bacterial isolation

Bacterial isolation and purification. Enrichment techniques were used to isolate bacteria from wastewater. Firstly, MSM and 10 mL of wastewater and sludge sample was added to a 250 mL sterilized conical flask. Prior to adding MSM to the flask, 10 mg L^{-1} of BZP was added as a carbon source. Following the addition of BZP to MSM, the mixture was incubated for seven days at 30 °C under aerobic conditions (150 rpm). Then, 1 mL of MSM was added to the freshly produced media. After three successful media transfers, 0.1 mL of the enrichment culture was applied using the dilution plate technique to an MSM agar plate containing 10 mg L^{-1} of BZP. The culture was spread on the agar plate using an L-shaped sterilized glass spreader. Seven dilutions (10–1–10–7) were prepared and incubated at 30 °C for 48 h. About 150 actively growing bacterial colonies from agar plates amended with BZP (10 mg L^{-1}) were obtained. Isolates from all inocula that had potential to grow on BZP amended MSM agar plates were purified by streaking twice on agar media and were selected for the screening experiment to check their potency for degradation.

Primary screening. In total, 150 distinct bacterial isolates were selected for the screening experiment and evaluated based on their ability to produce biomass, indicating the efficient growth of bacterial cells in the BZP-amended media. The ability of the strains to grow on liquid medium was assessed at BZP concentrations ranging from 10 to 1000 mg L^{-1}, and temperature of 30 °C for 48 h in a shaking incubator at 150 rpm. The amount of biomass that increased over time in the broth MSM was measured at regular intervals to assess the growth of bacterial isolates on the MSM-amended BZP (O.D value = 0.5). BZP-amended MSM without bacterial culture was used as a control. Five bacterial isolates were selected based on the increase in cell biomass at regular intervals through growth in liquid MSM amended with BZP (O.D value = 0.5) at an elevated BZP concentration (1000 mg L^{-1}). The primary screening was done based on the increase in cell biomass by calculating OD in BZP-enriched medium.

Secondary screening. For the screening of the most efficient bacteria, five bacterial isolates were grown in glass vials containing 10 μL of overnight culture grown in 10 mg L^{-1} of BZP-amended MSM broth (O.D 0.8 ± 0.03). Serum bottles containing 100 mL MSM were inoculated with bacterial cultures spiked with 20 mg L^{-1} of BZP and incubated at a temperature of 30 °C and pH = 7 for 120 h in a shaking incubator (150 rpm) to provide aerobic conditions. GC-MS was used for the measurement of BZP degradation during the secondary screening process. % Degradation was measured at regular intervals of 12 h. Detoxification was calculated using the method reported in ref. 32 and an Agilent GC-MS 7890 B (Limit of detection = 0.1 mg L^{-1}). Two bacterial species, DK2 and S4, were selected based on the cell biomass and BZP degradation rate. The two species were compared both singly and in consortia for degradation rate and further experiments. As a control, culture medium-enriched with BZP but without bacterial inoculum was kept. Considering the OD and percentage degradation, two efficient bacterial strains (DK2 and S4) were identified. Percentage degradation was calculated using eqn (1), as follows:

$$\text{BZP biodegradation}(\%) = \frac{\text{BZP control} - \text{BZP sample}}{\text{BZP control}} \times 100 \quad (1)$$

Optimization of the environmental conditions

Optimization of the process factors including pH, temperature, incubation duration, carbon and nitrogen source, and concentration was done for enhanced BZP degradation using the most effective bacterial species, DK2 and S4. A summary of the investigated parameters is presented in Table 1. NaOH and HCl (2 M) were used to adjust the pH of the media. After that, serum

Table 1 Summary of the parameters used in the optimization process

Parameter	Acronym	Unit	Values investigated
pH	—	—	5, 6, 7, 8, 9
Temperature	Temp	°C	25, 30, 35, 40, 45
Glucose concentration	$C_6H_{12}O_6$	g L^{-1}	2, 4, 6
Yeast extract concentration	—	g L^{-1}	2, 4, 6
Sucrose concentration	—	g L^{-1}	2, 4, 6
Ammonium nitrate concentration	NH_4NO_3	g L^{-1}	1, 2, 3
Urea concentration	CH_4N_2O	g L^{-1}	1, 2, 3
Potassium nitrate concentration	KNO_3	g L^{-1}	1, 2, 3
Benzophenone concentration	BZP	mg L^{-1}	20, 25, 30, 35

bottles containing 30 mL of MSM were sterilized for 20 min at 121 °C, and the pH was adjusted accordingly. 1 mL of bacterial cultures with an OD600 of 0.80 was added to the flask media as an inoculum. After determining the optimum pH, the impact of temperature was examined by varying it from 25 °C to 45 °C using the same culture method as previously described for pH. In the serum bottles, sterilised mineral salt medium (30 mL) with a pH of 7.0 and a final concentration of benzophenone according to the experimental setup were used. After that, one millilitre of bacterial culture (OD600 = 0.80) was added to the flasks. All inoculation flasks were left to incubate at 25 °C, 30 °C, 35 °C, 40 °C, and 45 °C. Abiotic degradation was also tested using uninoculated flasks. For 120 h, triplicate samples were cultured. The degradation of benzophenone at various temperatures was measured through GC MS. Then, the impact of carbon and nitrogen sources on BZP biodegradation utilizing DK2 and S4 was examined.

The concentrations of the three different carbon sources, yeast extract, glucose, and sucrose, were varied between 2 and 6 g L^{-1}, while the concentrations of the nitrogen sources, ammonium nitrate, urea, and potassium nitrate, were adjusted between 1 and 3 g L^{-1}. Likewise, by varying the concentration of BZP from 20 to 35 mg L^{-1}, the effect of its initial concentration on its biodegradation was evaluated. Using flasks that had not been inoculated, abiotic degradation was also examined for comparison. Using orbital shakers (Shelab) set to 0 rpm (stationary) and 150 rpm, the impact of agitation following incubation on bacterial species development and BZP biodegradation was investigated. The incubation time was selected as, 24, 48, 72, 96 and 120 h, respectively. The expansion of growth of bacterial communities was assessed based on the colony forming unit (CFU). The experiments were performed in triplicates and reported as mean.

Biodegradation assays

Sample preparation for GC-MS. Two of the most efficient bacterial strains (DK2 and S4) were chosen, and their degradation potential was determined. The experiment was run in triplicate in 100 mL of MSM and loaded with 20 mg L^{-1} of BZP sealed and kept in a shaking incubator at 150 rpm to achieve aerobic conditions. The biodegradation of BZP was monitored using GC-MS. The sample was prepared by extracting BZP from aqueous solution using ethyl acetate. Then, 2.5 mL of ethyl acetate was spiked into 5 mL of MSM and liquid–liquid separation was carried out through vortexing of the sample for 2 min. Na_2SO_4 was added to the organic fraction of the mixture in a beaker to eliminate any remaining moisture after the organic layer was separated from the aqueous phase. The organic layer was then transferred to a 50 mL sterile vial and dried using a nitrogen evaporator (N-EVAP 111, Massachusetts). The residues were then diluted with 1 mL of methanol for GC-MS analysis. Phenanthrene-d10 (10 μL of 10 mg L^{-1} solution in CH_3OH) was used as the internal standard.[32] Simultaneous analyses were also performed on the control samples. Mineral salt media were spiked with BZP without adding the respective bacterial strains DK2 and S4, which were important for comparing the results.

GC-MS analysis – an. Agilent 7890B gas chromatograph with a mass selective detector (Agilent 5975C) was used to determine the BZP concentration.[32] For separation and analysis, an HP mass column (30 m × 0.25 mm × 0.25 μm) was used. The flow rate of the carrier gas, helium, was set at 1 mL min^{-1}. Splitless mode was used for sample injection. The injector temperature was set at 280 °C. The temperature of the GC column was first set at 100 °C. It was then ramped up to 210 °C at a rate of 8° C min^{-1}. Finally, the temperature was accelerated at a rate of 25°C min^{-1} to 290 °C and maintained at this temperature for 8 min. The contact was constantly maintained at 280 °C and the mass spectrum was obtained. The samples were examined using selective ion monitoring. It was set to monitor the intensity of specific *m*/*z* values. To prepare a calibration curve, calibration standards were prepared at six levels, *i.e.*, 1 mg L^{-1}, 20 mg L^{-1}, 40 mg L^{-1}, 60 mg L^{-1}, 80 mg L^{-1}, and 100 mg L^{-1}. The difference in peak area and response factor at different retention times was analyzed and interpreted as percentage degradation of BZP. The samples were analyzed for the identification of BZP metabolites concerning the process of BZP degradation by the culture *Bacillus cereus* (DK2) and *Bacillus pumilus* (S4). Metabolites at an interval of 12 h were extracted and identified by gas chromatography-mass spectrometry (GC-MS), as described by Tang *et al.*[33] Additionally, during the degradation process, seven significant degradation products (Fig. S2) were identified based on the mass spectrum *m*/*z* values and relative abundance using the corresponding standard compounds from the National Institute of Standards and Technology (NIST) library database (match quality/percentage thresholds used for compound identification was above 900).

BZP degradation kinetics

Using pseudo-first-order (PFO1) and pseudo-second-order kinetic models, also referred to as the Lagergren rate equation, the rate of biodegradation of BZP by DK2 and S4 was ascertained.[34] Eqn (2) (PFO1) and (3) (PSO2) were used to estimate the parameters of the kinetic models. To simplify the process, the biodegradation kinetics was computed as the BZP concentration decreased.

$$\ln(q_e - q_t) = \ln q_e - \left(\frac{K_1 t}{2.303}\right) \quad (2)$$

$$\frac{T}{t_q} = \left(\frac{1}{K_2 q_2^2}\right) + \left(\frac{t}{q_e}\right) \quad (3)$$

where q_e and q_t are the degradation of BZP at equilibrium (e) and at any given time (t) and K_1 (h^{-1}) and K_2 (mg h^{-1}) are the rate constants of the PFO1 and PSO2 models, respectively. Kinetic modeling of biodegradation is significant to predict microbial reactions and regulate biological processes and transformation of substrates in municipal wastewater.

Gene sequence analysis (16S rRNA)

Bioinformatic analysis was performed for the two most efficient bacterial isolates (DK2 and S4) that were identified through primary and secondary screening. The 16S rRNA bioinformatic analysis was carried out according to.[35] It is common practice to compare almost whole 16S rRNA gene sequences to ascertain the taxonomic connections of bacterial strains; nonetheless, 98.65% similarity is currently acknowledged as the upper bound for species delimitation.[36]

Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene using 27F/1492R primers. The Thermal Scientific Gene JET Gel DNA Isolation kit (Thermo Fisher Scientific) was used to isolate bacterial DNA, which was then preserved at −20 °C. The collected DNA was purified using a Thermo Scientific GeneJET Gel DNA Purification kit before being sequenced by Macrogen Laboratories in South Korea. The DREAM Taq TM Green PCR Master Mix was utilised to create the solution for PCR analysis. Reverse (R) and forward (F) primers, Dream Taq TM, Green PCR Master Mix, DNA-free water, and a DNA template were all included in the PCR reaction mixture. Firstly, sequenced reads were assembled in SeqMan (Lasergene, DNAStar Inc., Madison, USA). The consensus contig was saved and similarity checked by BLAST in the NCBI database. Using closely related bacterial sequences that were found in the National Centre for Biotechnology Information (NCBI: **https://www.ncbi.nlm.nih.gov/**) databases by performing BLAST searches for bacteria, a neighbor-joining phylogenetic tree was constructed using reference sequences of different species in the Bacillaceae family and the top six hits of BLAST of the current isolates (DK2 andS4) sequence (Saitou and Nei, 1987). Multiple sequence alignment (MSA) was carried out using the MUSCLE program in the MEGA7 software.[37] The bootstrap method was used with 1000 replications and percent bootstrap values (less than 50% are hidden) are shown next to each branch.[38]

Statistical analysis

Experiments were designed in Completely Randomized Design (CRD). Using this design, treatments are assigned fully at random such that every unit of experimentation gets an equal chance to receive one treatment. Each experiment was conducted in three replications and data is presented as the mean of the replications. The wastewater quality metrics were found to have mean values, ranges, and standard deviations. Graph Prism 8 and MS Excel 7 were used to visualize the information and findings. One way ANOVA was used to analyze the wastewater characteristics to check if there was a significant difference in the means among the selected sites (p-value 0.05).

Results and discussion

Wastewater characteristics

Various characteristics including pH, COD, TDS, EC and BZP concentration were determined in the wastewater samples collected from different locations in Rawalpindi and Islamabad, displayed in Table S1. The average pH of the wastewater samples taken from the industrial area (SOP) was higher (pH = 7.9) than that of the other samples (7.8, 6.98 and 6.9). The alkaline pH of the samples taken from the SOP may reduce the efficacy of chlorine treatment and disinfection.[39] The samples from Noor colony (NC) (pH = 7.8) and Sohan (S) have pH values that were comparable (pH = 7.8) to that from the industrial area (pH = 7.9). However, the Dhoke Kala Khan (DK) (pH = 6.98) and Nulla Lai (NL) (pH = 6.9) samples had pH lower than all the other samples.

It important to note that all the wastewater samples had pH values within the range of 6–9, where bacterial strains could effectively degrade the targeted contaminants.[23] However, the average values are reported here, and thus the properties of the individual wastewater samples may differ, as displayed in Table S1. There was a statistically significant difference (p value < 0.05) in the six wastewater samples (SOP 2, NL5, NC5, S2, DK5, and DK4), while the other samples were not significantly different from each other.

The sample obtained from DK had the greatest average EC, measuring 263 μS cm^{-1}. Surprisingly, NL had the lowest average EC (146 μS cm^{-1}), despite receiving water from several municipalities. Previous studies reported elevated levels of EC (1200 μS cm^{-1}) in Nulla Lai.[40] The samples from NL displayed the lowest TDS concentration, but the average concentration in this particular site (DK) was determined to be higher at 209.33 mg L^{-1}. Lower EC and TDS levels in NL have been associated with seasonal fluctuations in rainfall pattern, as increased rainfall can dilute water and decrease the EC and TDS levels.[73] Moreover, there was a large variation in the TDS values of the municipal and industrial wastewater samples, where the sample with the highest TDS (NC5) had the highest pH. TDS ranged from 109.8 to 327.7 and there was a statistically significant difference (p value < 0.05) in the same wastewater samples having the above-mentioned pH values. The electric conductivity (EC) values also showed a significant difference (p value < 0.05) in all the wastewater samples and ranged from 115.13 to

340. Alternatively, it was observed that the samples from DK had the lowest average COD value at 221.71 mg L^{-1}, while NC had the highest COD at 482.67 mg L^{-1}. The maximum chemical oxygen demand (COD) value was 482.67 and observed in the same wastewater sample. However, the COD values ranged from 153.3 ± 7.07 to 482.67 ± 10.60, and except for three samples (DK2, S4 and SOP5), all others were significantly different (p value < 0.05) from each other, as indicated by the one-way ANOVA statistical test analysis. A comparison of the wastewater characteristics with the threshold values/permissible limit of NEQS Pakistan, WHO and FAO is shown in Table S1, which revealed that most of the municipal wastewater samples have COD levels higher than the threshold limit. Other parameters including pH, EC, TDS were below the permissible limits (NEQS, 2000 and WHO, 2006).

The levels for the wastewater characteristics under investigation were lower than the WHO recommended criteria for municipal wastewater, as reported by Alhaj *et al.*[41] This also indicated that the municipal wastewater samples were significantly contaminated, which promoted bacterial growth and proliferation. S and DK had the highest average BZP concentrations, measuring 16.5 and 18.0 mg L^{-1}, respectively, while NC had the lowest (11 mg L^{-1}). The samples taken from SOP and NL did not contain BZP (measured through GC MS and detection limit = 0.1 mg L^{-1}). The BZP concentration in the samples collected from Rawalpindi, Pakistan was higher than the reported values of 68.5 ng L^{-1} to 5.01 × 10^3 ng L^{-1} in surface water and the concentration of BZP in a wastewater treatment plant of 6.21 × 10^{-4}–9.51 × 10^{-4} ng L^{-1} in Shanghai, China (measured through LC MS/MS).[42] In the present work, the quantification of BZP was performed mainly because (1) GC-MS equipment was readily available in the university's laboratory facilities where this study was carried out and (2) GC-MS has reportedly been used by numerous comparable studies for the quantification of BZP in UV filters.[75,76] The benzophenone concentration was as high as 18 mg L^{-1}, which is above the permissible limit of 100 μg L^{-1} (US MDH, 2018). BZP cannot be easily degraded in the environment, and therefore it tends to bioaccumulate in aquatic organisms, and ultimately biomagnify in the food chain in ecosystems.[43]

Isolation of bacterial strains

A total of 150 bacterial strains was isolated from 25 wastewater samples taken from both municipal (DK, NL, NC, and S) and industrial areas (SOP). The distribution of the bacterial strains is shown in Table S2. Approximately 86% of the isolates were identified in municipal wastewater samples, and 14% were isolated in the effluent of SOP. The isolates were tested for their capacity to produce biomass in BZP-amended media at various initial concentrations after being purified using an enrichment process. Among the 150 isolates, only five strains (3.3%) were able to produce biomass (Table S3) according to the bacterial OD600 data ranging from 0.2 to 0.6, at different concentrations (10–1000 mg L^{-1}) after 48 h of incubation.

Most notably, the bacterial strains isolated from SOP were not able to grow in the presence of BZP. Following the first screening, the two most effective bacterial isolates (DK2 and S4) that produce biomass at an elevated concentration (1000 mg L^{-1}) of BZP and their ability to biodegrade BZP at 20 mg L^{-1} of concentration in broth media under aerobic conditions were identified.

Characterization of efficient bacterial isolates

The most efficient BZP degrading bacteria (DK2 and S4) were characterized by cellular growth, morphological, physiological and biochemical parameters, and phylogenetic tests.

Cellular growth. The cellular growth parameters are summarized in Table S3. Following the introduction of the bacteria into MSM enriched with 20 mg L^{-1} of BZP as the sole carbon source, the bacterial cell population displayed specific growth dynamics, including a lag period, during which DK2 and S4 began to adapt to their new environment. S4 acclimated to their new environment faster than DK2, as seen by the lag phase of 3.5 h for S4 and 4 h for DK2.

It was found that DK2 and S4 had similar doubling time, of 1.7 hours. For each isolate, the stationary phase lasted 72 hours. In the case of DK2 and S4, they showed bacterial growth of 1.0 × 10^5 ± 10^3 and 1.0 × 10^6 ± 10^2 CFU mL^{-1} after 72 h, respectively, indicating that organic compounds like BZP can act as sources of carbon and energy for the growth of DK2 and S4, as shown in Table S4. Fig. S3 displays the phylogenetic tree of the bacterial isolates DK2 (PQ380138) and S4 (PQ380140) with closely related species of the genus *Bacillus cereus* (DK2) and *Bacillus pumilus* (S4). The results suggested almost complete removal of BZP in 120 h with an increase in the number of bacterial cells.

Morphology and physiology. The morphological and physiological characteristics of DK2 and S4 are listed in Table S4. Both bacterial strains were rod-shaped with raised structure. The margins of the DK2 were square, whereas those of S4 were coiled, with both strains showing translucent opacity. The DK2 pigmentation was cream, whereas that of S4 was white. The growth pH and temperature range was 6.0–7.0 and 4.0–9.0 and 4–55 °C and 5–55 °C for DK2 and S4, respectively. Both the strains were NaCl tolerant.

Biochemical characteristics. The biochemical characteristics of DK2 and S4 are presented in Table S5. DK2 was catalase and Voges–Proskauer (VP) positive, whereas S4 was protease and lipase positive.

Phylogenetics. The phylogenetic tree was rooted using the *Emergencia timonensis* isolate (accession number = NR_144737) as the out-group (selected based on sufficient similarly to the in-group to make it possible for meaningful comparisons). The isolates from the current study were labelled with green circles. One isolate developed a separate branch in the group of *Bacillus* genus (shown in red-colored branches), which is very close to *Bacillus cereus* species with accession number DK2 (PQ380138) and *Bacillus pumilus* species with accession number S4 (PQ380140), as shown in Fig. S3(a and b), respectively.

Biodegradation of BZP

After the isolation and identification of the pure bacteria cultures (DK2 and S4), they were used to elucidate the

degradation mechanism and the biochemical pathways using minimum salt medium (MSM) with BZP as the sole source of carbon and energy for the bacteria. In this testing system, organic carbon was the only source, and thus any decrease in concentration with proper controls indicates that microbial degradation is directly coupled with the growth of microorganisms to establish a close link between a bacterium and the mineralization of the BZP.[44] DK2 and S4 were used to investigate the biodegradation of BZP (as the sole carbon source) both individually and in consortia at a constant initial concentration, agitation speed, temperature, and pH value of 20 mg L^{-1}, 150 rpm, 30 °C, and pH 7, respectively. The biodegradation of BZP is depicted in Fig. 1. Significant degradation of BZP was observed by selected strains from 0–120 hours. In the first 72 h, DK2 and S4 degraded more than 60% of BZP. DK2 showed 67.5% degradation, while S4 showed 69.5% degradation. The consortium (DK2 and S4) showed 86% degradation after 72 hours of incubation in liquid MSM under aerobic conditions. Degradation behaviour was accompanied by growth (CFU mL^{-1}) after 120 hours. The findings indicated that the concentration of BZP decreased as the density of bacterial cells increased, indicating that BZP promoted cell growth.

At 0 h, the CFU of DK2 and S4 was measured to be 0.19–0.2 cells per mL, both containing 20 mg L^{-1} concentration of BZP in MSM. After 24 h, the concentration of BZP was reduced to 14 mg/L and 16 mg L^{-1} by DK2 and S4, resulting in the formation of benzophenone dimethyl ketal and phenyl cyclohexyl ketone, respectively, with 0.42 CFU/mL of bacterial cells present. After 72 h, the bacterial cells increased to $1.0 \times 10^5 \pm 10^3$ and $1.0 \times 10^6 \pm 10^2$ CFU mL^{-1} with 6 mg L^{-1} and 4 mg L^{-1} concentration of BZP in MSM, which formed ethyl-3-(2-thienyl) propenoate and 2-cyclohexen-1-one, respectively. Moreover, after 120 h, the bacterial cells increased to $1.0 \times 10^7 \pm 10^2$ and $1.0 \times 10^8 \pm 10^3$ CFU mL^{-1} with 2 mg L^{-1} concentration and 1 mg L^{-1}, respectively, of BZP in MSM, resulting in the degradation products thiobenzoic acid, *O*-methyl ester and heptacosane. After 120 h of reaction, it was observed that both bacterial isolates (DK2 and S4) had eliminated BZP (20 mg L^{-1}) in liquid MSM.

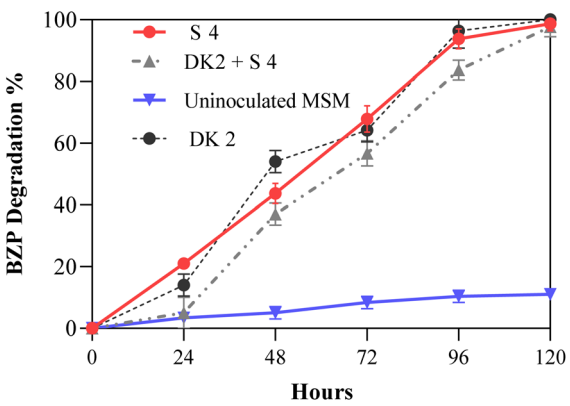


Fig. 1 Biodegradation of benzophenone (BZP) (as the sole carbon source) by bacterial isolates under aerobic conditions. Experimental conditions: BZP concentration: 20 mg L^{-1}, pH: 7; temperature: 30 °C, agitation speed: 150 rpm, and incubation duration: 120 h.

The consortium DK2 + S4 degraded BZP significantly (p value < 0.05) (17% to 19%) more than individual bacterial isolates (DK2 and S4) after 72 h of incubation in liquid MSM. Previously, other studies also reported that consortia of bacteria were effective in the biodegradation of organic compounds (4-methylbenzylidene)camphor (4-MBC) and 2-ethylhexyl 4-(dimethylamino)benzoate (EH-DPAB), two of the most frequently used UV-filters.[45] Even after 120 h of reaction time, less than 10% of BZP could be degraded by the uninoculated MSM.

An earlier investigation using *Methylophilus* sp. FP-6 reported 15% degradation of 5 mg L^{-1} of BP-3 in 8 days of incubation without the addition of methanol, whereas adding 120 mg L^{-1} methanol as the carbon substrate enhanced the degradation to 50% in just 4 days. Further optimization of the experimental conditions (culture temperature of 40 °C, inoculum rate of 4%, shaker speed of 150 rpm, and neutral initial pH of 7) led to 65% degradation of BZP in 8 days of incubation.[23] In the current investigation, the efficiency of BZP biodegradation by DK2 and S4 also surpassed the previously reported values of 26.7% and 14.7% in 24 h of reaction using *Staphylococcus aureus* and *Salmonella typhimurium*, respectively.[43] In contrast to *Serratia rubidae*, which could only degrade 12.7% of 4-hydroxybenzophenone in 24 h, *Salmonella typhimurium* could degrade double (24%) that of *Serratia rubidae*.[43] The degradation efficiency of BZP in the current study was 35% higher than that reported in another study (approximately 40% ± 5% degradation of benzophenone-3 (BP-3) after 8 days of incubation under aerobic conditions).[46]

The most likely causes of the discrepancy in performance are the different isolates, each of which may have a different capacity to degrade BZPs, and the differences in chemical structure and functional genes.[47] In the previous study, BZP-3, a derivative of BZP, was biodegraded by *Methylophilus* sp. FP-6,[23] whereas in the current work, BZP was biodegraded by *Bacillus cereus* (DK2) and *Bacillus pumilus* (S4). As a result, each compound may need different treatment conditions, or it would be the different initial concentrations, which in the current study was 20 mg L^{-1}, whereas in the prior study, it was 10 mg L^{-1}. Microorganisms that selectively degrade a particular pollutant can grow and reproduce physiologically when the concentration of that pollutant in the environment or incubation culture medium is higher than the maintenance threshold of 1 μg mL^{-1}.[48] However, the microbial population decreases and is unable to effectively degrade the pollutant as the concentration of the pollutant increases to a point where toxicity is apparent. As a result, enrichment in a laboratory environment or on-site bioremediation cannot yield the desired positive results.[49] Herein, the concentration of BZP was chosen in such a manner that falls between maintenance and toxicity to achieve metabolism

Chemical structure, polarity, the type and number of substituents on the aromatic ring, and the concentration of the compounds have all been shown to impact biodegradation.[50] For example, when testing phenolic compounds (4-hydroxybenzoic acid and catechol) at the same concentration, the efficiency of *Acinetobacter* sp. PK1 and *Methylobacterium* sp. NP3 varied significantly, and this variation in efficiency could be

explained by the differences in the nature and structure of the compounds tested.[51] Alternatively,[52] reported more than 90% of biodegradation of BZP-3 in 24 h using an expanded granular sludge bed (EGSB) reactor. As previously pointed out by,[27] many environmental factors, such as pH, temperature, moisture, and nutrient availability, might affect the susceptibility of bacterial isolates. Furthermore, the type and concentration of contaminants in their environment can have an effect on the viability and growth of microorganisms. As a result, this study explores the impact of some of the most significant parameters or environmental factors on the biodegradation of BZP using the DK2 and S4 isolates. Then, the results are thoroughly presented and discussed in the following section.

Kinetics of biodegradation of BZP

Kinetic studies are important for regulatory purposes because they may be used to forecast the degree of biodegradation at different times, especially the extent of biodegradation and adjustment to a model for the development of ultimate treatment systems on an industrial scale. Kinetic studies were carried out and the kinetic parameters, *i.e.*, q_e, q_t, K_1, K_2 and R^2, were estimated both in the case of PFO1 and PSO2, as indicated in Table 2 and Table 3, respectively. The kinetic plots are depicted in Fig. 2. The findings showed that the biodegradation of BZP by the DK2 bacterial isolate followed PSO2 with a higher coefficient of determination ($R^2 = 0.99$ with a rate constant of 0.94), indicating that the pseudo-second-order model (PSO2) is suitable for modelling DK2 in the degradation of benzophenone; however, S4 followed PFO1 with a higher coefficient of determination ($R^2 = 0.98$ with a rate constant of 0.8) than PSO2 ($R^2 = 0.87$), indicating that the pseudo-first-order model (PFO1) is suitable for modelling in the S4 degradation of benzophenone. The R^2 between 0 and 1 measures how well a statistical model predicts the degradation of organic compounds. PSO2 best fitted DK2, while PFO1 was utilized for the S4-assisted biodegradation of BZP, as shown in Table S6.

At 120 h, the S4 kinetic rate constant (K_1) increased gradually throughout the course of each degradation, reaching a maximum of ($K_1 = 0.84$/h). Alternatively, the kinetic rate constant (K_1) for DK2 was approximately 0.94/h for the entire period of monitoring. This result could be explained by the differing enzymatic activities of these two distinct bacterial isolates, even though they were both exposed to the same BZP concentration (20 mg L^{-1}). A previous study showed the degradation of 3-(4-methylbenzylidene) camphor, a frequently used UV-filter in various personal care products, with kinetic rate constants of 0.0020–0.039/day. In contrast to our study, the kinetic rate constants for both species (DK2 = 0.94 and S4 = 0.84/h) were higher than that of 0.0020–0.039/day under aerobic conditions reported for 3-(4-methylbenzylidene)camphor through microcosm studies on biodegradation in marine sediments sampled from two sites in Italy using a consortium of microorganisms.[45] In the case of DK2 and S4, the PFO1 and PSO2 biodegradation kinetic rate constants of BZP are also greater than the kinetic rate constant of 0.025 when utilizing a UV light with an intensity of 900 μW cm^{-2}, an H_2O_2 concentration of 0.30 mmol L^{-1}, and an initial BZP concentration of 22.5 mg L^{-1}.[53] Furthermore, compared to the UV/H_2O_2 system,

Table 2 Pseudo-first-order (PFO1) and pseudo-second-order (PSO2) kinetic model parameters for the degradation of BZP by *Bacillus cereus* (S4) using the Lagergren rate eqn (2) and (3), respectively

Time (h)	Concentration (mg L^{-1})	q_t	Log ($q_e - q_t$)	t/q_t
0	20	37.6	1.301	0.00
24	16	41.6	1.204	0.577
48	08	49.6	0.903	0.161
72	04	53.6	0.602	1.343
96	02	55.6	0.301	1.720
120	00	57.6	0.00	2.080

Table 3 Pseudo-first-order (PFO1) and pseudo-second-order (PSO2) kinetic model parameters for the degradation of BZP by *Bacillus pumilus* (DK2) using the Lagergren rate eqn (2) and (3), respectively

Time (h)	Concentration (mg L^{-1})	q_t	Log ($q_e - q_t$)	t/q_t
0	20	37.6	1.301	0.000
24	14	43.6	1.1406	0.500
48	10	47.6	1.000	1.008
72	06	51.6	0.778	1.390
96	02	55.6	0.301	1.727
120	00	57.6	0	2.083

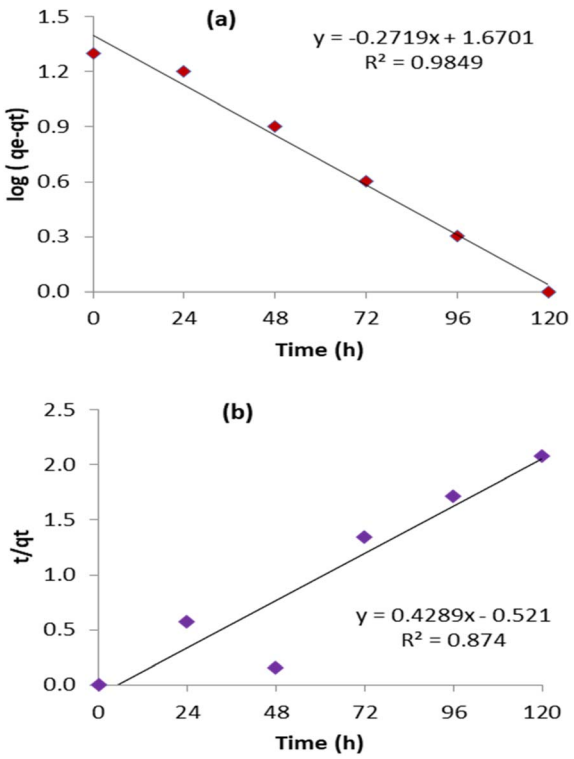


Fig. 2 Plot of log($q_e - q_t$) *vs.* time of (a) *Bacillus pumilus* (S4) and *Bacillus cereus* (DK2), and graph of log(t/q_t) *vs.* time of (b) *Bacillus pumilus* (S4) and *Bacillus cereus* (DK2) for determining the reaction order of the biodegradation of BPZ.

the BZP degradation efficiency of 100% at 20 mg L^{-1} was higher than the reported value of 74.9% at 13.75 mg L^{-1}.

However, the rate of BZP degradation in this study was much slower than in the previous one.[53] In this study, 100% of BZP was biodegraded within 120 h, whereas in the prior study, 74.9% was degraded in just 30 min of UV irradiation. Overall, the results show that DK2 and S4 have promising potential for biodegrading the BZP present in personal care products. These microbial isolates (DK2 and S4) provide a potential ahead for tackling the complex issue of personal care product pollution through the analysis of important parameters and the understanding the pathway for the degradation of organic compounds.

Influence of process factors on BZP biodegradation

Few studies have examined the biodegradation of BZP in municipal wastewater and assessed how different environmental factors, such as temperature, pH, sources and concentrations of carbon and nitrogen, and BZP initial concentration, affect the degradation of BZP by DK2 and S4. In bioremediation processes, it is essential to optimize environmental parameters including moisture content, oxygen levels, temperature, nutritional supply, and pH to stimulate microbial growth and pollutant degradation.[27] The selected isolates and optimal conditions would be used as the basis for the larger-scale design of a biotreatment system for wastewater contaminated with BZP. Sustainable and effective microbiological remediation solutions depend on precise pollutant quantification and comprehension of site-specific features.[54] Therefore, the impact of the most important distinct process variables on BZP biodegradation utilizing DK2 and S4 was assessed in this study, and the results are presented and discussed in detail in the following sections.

Effect of temperature on BZP biodegradation. Temperature affects the metabolism and activity of bacteria. The growth and metabolic rate of bacteria are enhanced by an optimal temperature.[55] Thus, using DK2 and S4, the impact of temperature variation on BZP degradation was assessed at 25 °C, 30 °C, 35 °C, 40 °C, and 45 °C. The temperature range of 25–45 °C is commonly used in laboratory experiments, as it represents the optimal growth temperatures for numerous mesophilic bacteria, including various *Bacillus* species, which may exhibit different optimal temperatures. Moreover, the temperature range of 25–45 °C serves as a standard range that covers both mesophilic and thermophilic growth.[77,78] Fig. 3(a) illustrates the impact of temperature on the biodegradation of BZP. Under all the experimental conditions, the DK2 and S4 isolates were able to degrade BZP, albeit to varying degrees. At 25 °C, less than 40% of BZP was degraded. This may be because the substrate moves through the DK2 and S4 cells less readily at lower temperatures. Low temperatures have long been thought to impede the uptake of important substrates due to their effects on the cell membrane as lipids inside the bacterial membrane could become harder, which reduces the efficiency of substrate degradation.[56] When the temperature was increased from 25 °C to 30 °C and 35 °C, the BZP degradation increased to more than

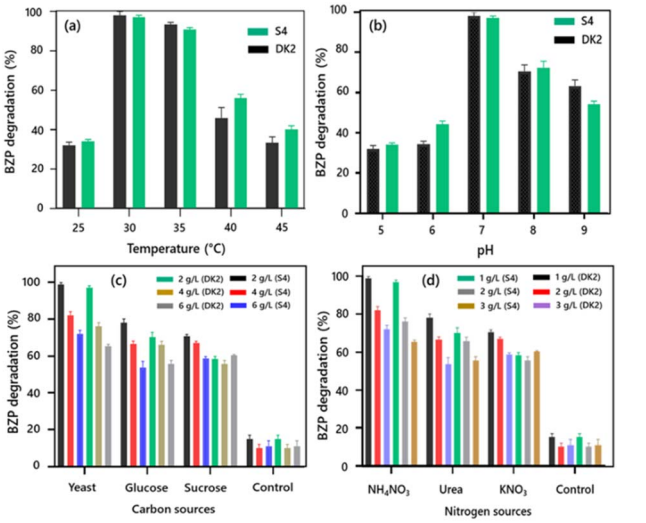


Fig. 3 Effect of various parameters on the biodegradation of BZP using DK2 and S4 bacterial isolates under anaerobic conditions: (a) temperature, (b) pH, (c) carbon source and concentration, and (d) nitrogen sources and concentration. BZP concentration 20 mg L^{-1} and incubation duration 96 h.

95%. BZP and its derivatives have considerably high boiling points (about 300 °C to 500 °C) and minimal vapor pressures (1.3×10^{-11} to 1.9×10^{-3} mm Hg) and exhibit low volatility, making their loss by volatilization negligible.[79,80] Evaporation of BZP does not occur at these temperatures (vapour pressure = 1 mm Hg (108 °C) (Chemical Handbook 2024). However, when the temperature was increased to 40 °C or 45 °C, the efficiency of BZP degradation decreased (<60%). The corresponding decrease in the metabolic activity of DK2 and S4 may be the reason for the decrease in the BZP degradation efficiency at higher temperatures (>35 °C). Thus, 30 °C was identified as the optimum temperature for the efficient degradation of BZP using the DK2 and S4 bacterial isolates isolated from municipal wastewater.

Effect of pH on BZP biodegradation. Following the determination of the optimal temperature, further investigations were carried out at different pH values of 5, 6, 7, 8, and 9 while maintaining a temperature of 30 °C and a BZP concentration of 20 mg L^{-1} to explore the effect of pH on the biodegradation of BZP by DK2 and S4. The effect of pH on the biodegradation of BZP is shown in Fig. 3(b). At neutral pH of 7, both the DK2 and S4 isolates were able to degrade more than 99% of BZP. The biodegradation of BZP decreased significantly as the pH moved away from neutral, either higher or lower. These results are consistent with previous research employing *Methylophilus* sp. strain FP-6, which showed that the optimum temperature for the 61.9% degradation of BZP-3 after 4 days of incubation was 35 °C and pH 7.[23] It has been shown that the majority of bacteria that degrade organic pollutants, particularly *Pseudomonas* and *Bacillus* species, function best in the pH range of 6 to 8. This may be because bacterial isolates with pH values below 6 and above 8 have lower total enzymatic activity (amylase and protease).[57]

Effect of carbon source and concentration on BZP biodegradation. This study assessed several carbon sources as energy sources such as yeast extract, glucose and sucrose, and examined how they affected the ability of DK2 and S4 to biodegrade BZP. The degradation of BZP by DK2 and S4 in the presence of various carbon sources is displayed in Fig. 3(c). The results show that after 96 h of incubation, unsupplemented DK2 and S4 could degrade less than 15% ± 2% of BZP. This is consistent with a prior study, which reports that even after 8-days of incubation, BZP-3 degradation was only 15% in the absence of a carbon source.[23] These findings showed that BZP could not be fully utilised as the sole carbon source for bacterial growth, leading to BZP degradation, and that the absence of a carbon source had a significant impact on the potential of the DK2 and S4 strains to degrade BZP. In addition to enabling bacteria to sustain cell viability, encourage their growth, and proliferate, providing them with an adequate carbon source also improves the activity of enzymes for the co-metabolism of organic compounds.[58]

In the current study, the degradation efficiency of BZP increased to about 100% ± 2% when yeast extract was added as a carbon source. The responses of both isolates to the addition of yeast extract are comparable. The findings showed that these bacteria isolates have improved ability to degrade BZP in the presence of 2 g L^{-1} yeast extract as co-substrates. Other carbon sources, such as glucose and sucrose, were less accessible for the bacteria than yeast extract, as evidenced by their lower degradation efficiency (<80% ± 3%). In contrast, in a previous study, the degradation efficiency increased to over 50% when methanol was utilized at a rate of 120 mg L^{-1} as a carbon source in the biodegradation of BZP-3, as opposed to only 30%, 12%, 18%, and 28% when glycerine, sucrose, glucose, and ethanol, respectively, were used as the carbon source.[23] Additionally, investigations show that the addition of glucose (2.5 g L^{-1}), an easily utilizable carbon source, resulted in the generation of metabolites that differed from the growth results where the only carbon source used was chlorophenoxy acid (esters and their salts are widely used as herbicides).[59]

Research demonstrated that the biodegradation of two types of BZPs, such as 3-(4-methylbenzylidene)camphor and 2-ethylhexyl 4-(dimethylamino)benzoate, which is driven by metabolism by the native microbial communities in sediments, was enhanced by the addition of methanol as a carbon source.[45] In contrast, *Rhodococcus* sp. USK10 has been shown to utilize BZP-3 as sole carbon source without the addition of an external carbon source.[24] Hence, previous studies demonstrated that co-metabolism by microbial communities using more easily-degradable carbon sources can lead to the accelerated degradation of certain organic pollutants like UV filters and herbicides.[60] Silicene-based quantum dots nanocomposite-coated functional UV-protected materials can be prepared for overall environmental protection and long-term health solutions.[81]

However, the so-called degradation in terms of concentration changes is more precisely referred to as co-metabolism to differentiate it from metabolism under manipulation.[61,62] Although the degradation process may be accelerated by the addition of external energy sources, the availability of various substrates makes it challenging to properly understand the underlying mechanism of microbial physiology and biochemistry from a scientific standpoint.[63] To synchronize bacteria at the same or very similar stage of growth and generation time, this aspect must be further examined and validated by microbial physiology and bioenergetic investigations.[49]

Not only was the source of carbon important, but also its concentration. When the concentration of yeast extract was increased to 4 and 6 g L^{-1}, the degradation of BZP was less than 80%, while it was around 100% at 2 g L^{-1} in 96 h of incubation. The efficiency of BZP degradation similarly declined as the concentration of other carbon sources increased above 2 g L^{-1}. Yeast extract at a concentration of 2 g L^{-1} was shown to be the most effective carbon source for the growth of DK2 and S4 bacteria and the subsequent degradation of BZP among the carbon sources and their concentrations. Bacteria are capable of using various carbon sources, which they utilize as building blocks during their synthesis. Moreover, carbon sources provide the majority of energy needed for bacterial metabolism.[64] However, any feasible supply of carbon and energy has a minimum concentration limit at which the number of viable cells remains relatively constant given that the newly formed cells are equivalent to the cells that died. Early studies tested this theory using *Escherichia coli* as a model bacterium, and the glucose threshold concentration in the chemostat fermentor was approximately 1 μg mL^{-1}.[48] According to reports,[49] this threshold is crucial for three reasons, as follows: (1) successful enrichment of the pollutant-degrading microorganisms can only be accomplished when the specific concentration used is higher than the maintenance requirement under the relevant environmental conditions; (2) the physiological state of the microorganisms is at a boundary condition, either to multiply when more substrate is available or to die when it becomes limited; and (3) effective bioremediation at any site also requires that the bioavailable concentration of the specific pollutant is above the maintenance level under the complex environmental conditions. In environmental microbiology, these considerations make this threshold concentration value essential for the degradation of organic contaminants. In addition to providing energy for the development and survival of microorganisms, carbon sources act as electron donors, which are required for breaking benzophenone bond. These electron donors also necessary for producing equivalent NADH and NADPH, which are transmitted to benzophenone during the degradation process.[65]

Effect of nitrogen source and concentration on BZP biodegradation. Nitrogen-derived compounds are essential components that comprise the cell walls of bacteria and play a major role in supporting microbial development. Nitrogen is also found in the primary structures of amino acids, organic acids, and DNA.[66] Experiments were carried out with varying nitrogen sources and concentrations while keeping the other parameters constant at 30 °C, pH 7, and yeast as the carbon source at 2 g L^{-1} to evaluate the impact of nitrogen sources and their concentration on BZP degradation by DK2 and S4. Fig. 3(d) depicts the effect of different nitrogen sources on BZP

degradation. The results show that the BZP biodegradation efficiency dropped below 20% from the initial 20 mg L^{-1} when no nitrogen source was supplied. This result is in agreement with another study, which found that after incubating *Klebsiella* sp. SQY5 for seven days without the addition of an external nitrogen source, the concentration of tetracycline only decreased by 41.6%.[67] BZP and tetracycline both are aromatic compounds present in pharmaceuticals and personal care products and belong to emerging pollutants that are frequently detected in surface water and municipal wastewater.[68]

When ammonium nitrate (NH_4NO_3) was introduced as a nitrogen source, the BZP biodegradation efficiency increased to around 100% ± 3% in 96 h of incubation. The BZP degradation efficiency was less than 80% for urea and potassium nitrate (KNO_3), two other nitrogen sources. This can most likely be attributed to the decreased biomass yield with the addition of potassium nitrate (KNO_3) and urea as nitrogen sources, indicating that potassium nitrate (KNO_3) and urea inhibit biomass yield at 2 g L^{-1} and 3 g L^{-1}, respectively. It is important to note that up to a specific concentration, such as 1 g L^{-1}, each nitrogen source enhanced the biodegradation of BZP using DK2 and S4. Even when using ammonium nitrate (NH_4NO_3) as a nitrogen source, the degradation efficiency decreased to <80% as the level of nitrogen source exceeded the level of 1 g L^{-1}. The results suggest that when the isolates were not supplied with additional external carbon and nitrogen sources, DK2 and S4 used BZP as a source of carbon and nitrogen. When added yeast extract was used as the carbon source, DK2 and S4 used BZP as a nitrogen source, while both isolates used BZP as a carbon source when NH_4NO_3 was used as the nitrogen source. Consequently, more research will be required for understanding how the carbon to nitrogen ratio affects the aerobic DK2 and S4 degradation of BZP.

Effect of initial concentration on BZP biodegradation. The organization of microbial communities in a biological treatment system has been shown to be influenced by the concentration of BZP along with other environmental factors (pH, temperature, and co-substrates).[52] The effect of initial concentration of BZP on its biodegradation employing DK2 and S4 was evaluated and the results are presented in Fig. 4. When the concentration of BZP was increased from 20 mg L^{-1} to 35 mg L^{-1}, as shown in Fig. 4(a), the biodegradation of BZP by DK2 dropped from 98% ± 2% to 70% ± 6% in 96 h of incubation.

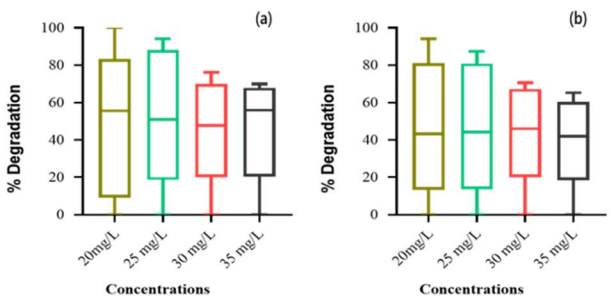


Fig. 4 Effect of initial BZP concentration on its biodegradation using (a) S4 and (b) DK2. Incubation time: 96 h, temperature: 30 °C, yeast: 2 g L^{-1}, ammonium nitrate: 1 g L^{-1} and pH: 7.

Applying S4, as shown in Fig. 4(b), resulted in a similar decrease in BZP biodegradation efficiency with an increase in BZP concentration. However, when the concentration of BZP was increased from 20 to 35 mg L^{-1}, the decrease in biodegradation efficiency of BZP by S4 was greater than that of DK2, as seen by the drop from nearly 100% to 78%. This suggests that the biochemical consumption of BZP by a selective microorganism is governed by its microbial physiology and biochemistry[49] and that the appropriate concentration range of both the chemical and the microorganism must be established before planning and carrying out the large-scale bioremediation of BZP.

Moreover, after 96 h, the bacterial cells increased (O.D 600 = 0.9) with 4 mg L^{-1} and 2 mg L^{-1} of BZP remaining in MSM at the initial BZP concentration of 20 mg L^{-1} for DK2 and S4, respectively. With an increase in the cell numbers (O.D 600 = 0.8) after 96 h, 6 mg L^{-1} and 4 mg L^{-1} of BZP remained in MSM at the initial BZP concentration of 25 mg L^{-1} for DK2 and S4, respectively. Moreover, in the same incubation period, the bacterial cells decreased (O.D 600 = 0.7) with 8 mg L^{-1} and 6 mg L^{-1} of BZP remaining in MSM at the initial BZP concentration of 30 mg L^{-1} for DK2 and S4, respectively. However, the bacterial cells further decreased (O.D 600 = 0.5) with 12 mg L^{-1} and 10 mg L^{-1} of BZP remaining in MSM at the initial BZP concentration of 35 mg L^{-1} for DK2 and S4, respectively. In contrast, the natural (solar irradiation) biodegradation of BZP (100%) after 12 days of treatment in a prior investigation at a lower concentration of 10 μg L^{-1} (ref. 21) occurred more slowly than the BZP degradation of 98% and 100% at 20 mg L^{-1} by DK2 and S4 in 96 h of incubation in the current work. It was also reported earlier that an increase in BZP concentration decreases the degradation efficiency. At a higher concentration of BZP, better degradation results were obtained in

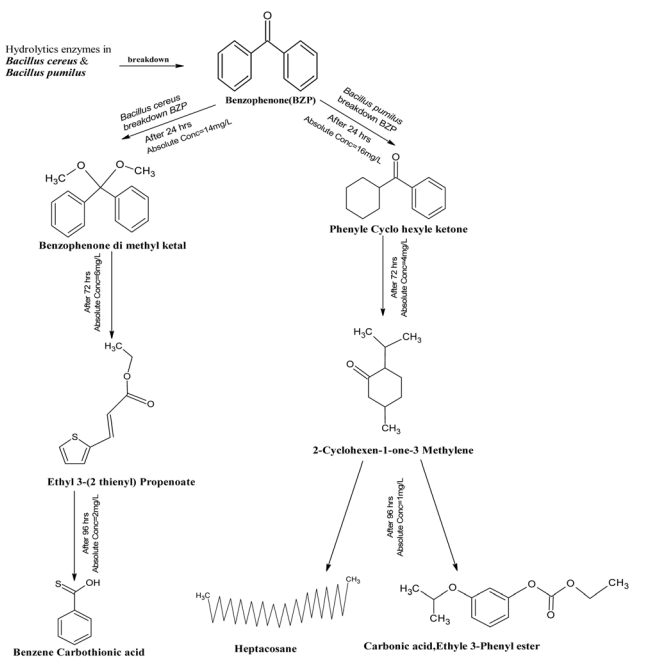


Fig. 5 Proposed mechanism for the degradation of benzophenone using selected isolates (DK2 and S4) under aerobic conditions.

our study because these bacterial strains are capable of surviving at higher concentrations of BZP.

Biodegradation mechanism and pathway for BZP. For the identification of the metabolites formed at regular intervals as a result of the degradation of BZP, MSM was taken after a 24 h period, and the metabolites were identified using gas chromatography (GC), coupled with MS. Their separation with their retention times was combined with mass spectrometry (MS) to determine the mass-to-charge ratio and fragmentation pattern of each separated metabolite, and then these data points were compared with the NIST library database using the NIST MS Search software. The degradation mechanism for BZP is explained by the structural scheme put forth by,[69] who stated that the chemical identification of the degradation intermediates and the construction of the degradation reactions and pathway can be used to study the transformation and degradation of pollutants.

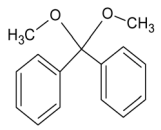
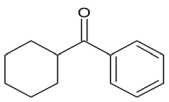
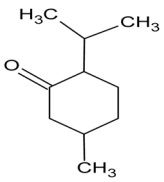
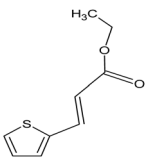
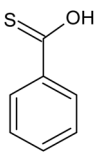
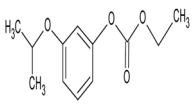
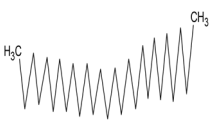
Based on the identification of the intermediates in the current work and previously reported results, the probable biodegradation mechanism of BZP employing DK2 or S4 is illustrated in Fig. 5. Within 72 h, four major compounds were detected and identified, and after 96 h, four more metabolites were identified, as shown in Table 3.

The DK2 and S4 isolates are comprised of hydrolytic enzymes, and thus it is possible that these enzymes helped degrade BZP into a simpler, less toxic intermediate during the biodegradation process.[23,25]

Monitoring the intermediate products of BZP degradation by DK2 and S4 allowed the detection of six distinct metabolites, as presented in Fig. S2. It was suggested that BZP transformed into benzophenone dimethyl ketal by strain *Bacillus cereus* after 24 h with a BZP concentration of 14 mg L^{-1} due to the demethylation of the methoxy substituent, while *Bacillus pumilus* degraded BZP in to phenyl cyclohexyl ketone with a BZP concentration of 16 mg L^{-1} (Fig. S2a).

There have been prior reports on the *O*-demethylation of BZP-3 ref. 23 and 25 and other organic compounds in both oxic and anoxic environments.[70] Next, after 72 h, the metabolites

Table 4 Chromatographic properties of benzophenone metabolites formed during degradation by DK2 and S4 strains and identified through GC-MS

Metabolite	Structure	Time (h)	Fragmentation ion (*m*/*z*)	Molecular weight
Benzophenone dimethyl ketal		24	105, 182, 77	288.28 g mol^{-1}
Phenyl cyclohexyl ketone		24	105, 188, 77	188.26 g mol^{-1}
2-Cyclohexen-1-one		72	110, 138, 152	96.127 g mol^{-1}
Ethyl-3-(2-thienyl) propenoate		72	137, 182, 109	198.24 g mol^{-1}
Thiobenzoic acid		96	121, 152, 77	138.18 g mol^{-1}
O-methyl ester		96	110, 82, 95	108.19 g mol^{-1}
Heptacosane		96	57, 71, 85	380.73 g mol^{-1}

from MSM were identified, and *Bacillus cereus* transformed dimethyl ketal into ethyl 3-(2-thienyl)-propenoate and identified with a BZP concentration of 6 mg L; however, *Bacillus pumilus* transformed phenyl cyclohexyl ketone into 2-cyclohexen-1-one-3 methylene (Fig. S2c).[71,72]

Eventually, after 96 h, *Bacillus cereus transformed* ethyl 3-(2-thienyl)-propenoate into the end-product benzenecarbothioic acid, which was detected with a concentration 2 mg L^{-1}, while *Bacillus pumilus* degraded 2-cyclohexen-1-one into heptacosane, carbonic acid, and O-methyl ester with a BZP concentration 1 mg L^{-1}. However, no evidence for this mechanism for the biodegradation of BZP-3 using *S. wittichii* BP14P was observed Table 4.[25]

When *Bacillus cereus* degraded benzophenone, the mass balance at the end of degradation was 98%. The metabolites formed during the first 72 h account for 70% mass compared to the total mass of benzophenone, while 28% mass formed by the rest of the metabolites in the next 24 hours. However, in the case of *Bacillus pumilus*, the mass balance at the end of degradation was 97%. The metabolites formed during the first 72 h account for 70% mass compared to the total mass of benzophenone, while 27% mass formed by the rest of the metabolites in the next 24 hours.

Conclusion

By demonstrating their demonstrated ability to degrade benzophenone under a variety of environmental conditions, the bacterial strains *Bacillus cereus* and *Bacillus pumilus*, with accession numbers DK2 (PQ380138) and S4 (PQ380140), respectively, isolated from municipal wastewater, their adaptability was highlighted. A single strain or consortia of both isolates could efficiently degrade benzophenone as the only carbon source, with the consortium demonstrating a significant degradation efficiency. Under the optimum environmental conditions of pH 7, temperature of 30 °C, yeast extract as the carbon source at a concentration of 2 g L^{-1}, ammonium nitrate as the nitrogen source at a concentration of 1 g L^{-1}, and benzophenone concentration of 20 mg L^{-1} in 96 h of incubation, both isolates were able to completely degrade benzophenone. It is possible to make active progress toward creating a more sustainable and cleaner environment by utilizing the complex metabolic abilities and enzymatic pathways of bacteria, as well as their applied biodegradation.

Author contributions

Ms Amna Maqsood did all the experimentation and prepared the initial draft. Dr Shahid Mahmood and Prof. Azeem Khalid supervised the research, provided funding resources and edited the manuscript. Prof. Irfan Aziz was a member of supervisory committee and helped with the data analysis. Dr Rab Nawaz and Dr Marlia Mohd Hanafiah edited the manuscript, prepared a sampling map, graphical abstract and the figure illustrating the possible mechanism diagram. Prof. Birthe Veno Kjellerup supervised Ms Amna Maqsood during her IRSIP scholarship at University of Maryland, USA, and especially degradation experiment was performed in her laboratory at University of Maryland, USA.

Conflicts of 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.

Data availability

Data will be made available on request.

Supplementary information (SI): the description map for the sampling sites, phylogentic trees, morphological and biochemical data of the selected strains, chromatographs of the degradation products *etc.* See DOI: **https://doi.org/10.1039/d5ra05131b**.

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