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ORIGINAL RESEARCH PAPER

Biodegradation of ketoprofen using a microalgal–bacterial consortium

Maha M. Ismail · Tamer M. Essam · Yasser M. Ragab · Fathia E. Mourad

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Abstract
Objective To test the toxicity of ketoprofen (a commonly-used NSAIDs) using two microalgal strains and *Artemia* sp. following the isolation of bacterial and microalgal strains and testing their ability to biodegrade and tolerate ketoprofen.
Results *Chlorella* sp. was the most resistant to ketoprofen. A defined bacterial consortium (K_2) degraded 5 mM ketoprofen as a sole carbon source both in the dark or continuous illumination. Ketoprofen did not undergo photodegradation. In the dark, biodegradation was faster with a lag phase of 10 h, 41% COD removal and 82 % reduction in toxicity. The consortium degraded up to 16 mM ketoprofen. The consortium was composed of four bacterial isolates that were identified. MS/MS analysis suggested a ketoprofen biodegradation pathway that has not been previously reported. Combining *Chlorella* sp. and the K_2 consortium, ketoprofen was degraded within 7 days under a diurnal cycle of 12 h light/12 h dark.
Conclusion The feasibility of using a microalgal–bacterial system to treat pharmaceutical wastewater is promising for the reduction of the process cost and providing a safer technology for pharmaceutical wastewater treatment.

Keywords *Artemia* · Biodegradation · *Chlorella* · Ketoprofen NSAIDs · *Spirulina* · Wastewater

M. M. Ismail (✉) · T. M. Essam · Y. M. Ragab · F. E. Mourad
Microbiology and Immunology Department and Biotechnology Center, Faculty of Pharmacy, Cairo University, Kasr Al-Aini Street, Cairo 11562, Egypt
e-mail: maha.ismail@pharma.cu.edu.eg

Introduction

A wide variety of persistent and toxic organic micropollutants are found in effluents from domestic and industrial wastewater treatment that can adversely affect human and public health. Attention has been dedicated to assess the behavior of pharmaceuticals and personal care products during the processes of wastewater treatment (Ziylan and Ince 2011). Verlicchi et al. (2012) reported that no legal limits are set for the discharge of pharmaceuticals into surface waters and a large portion of pharmaceuticals are excreted from human to animals either unaltered or as metabolites which, after disposal to municipal sewage treatment systems, can penetrate into groundwater. Some of the unfavorable impacts caused by drug pollution in environment involve aquatic life toxicity, development of microbial resistance, and disruption of endocrine function.

Non-steroidal, anti-inflammatory drugs (NSAIDs), such as Ibuprofen, ketoprofen, diclofenac and indomethacin, are considered the most predominant class of pharmaceuticals to be detected in the wastewater

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treatment plants at considerable levels ranging from 30 to 320 μg l^{-1}. The removal of many NSAIDs during municipal wastewater treatment is incomplete and residues of these compounds have been detected in surface waters up to several μg l^{-1}. For polar compounds, such as acidic pharmaceuticals, biodegradation is the most important removal process in activated sludge wastewater treatment (Kloepfer et al. 2004).

Microalgal/bacterial-based wastewater treatment is a promising approach where algae provide the O_2 required for bacterial degradation. Although the variation in the influent toxicity is a limiting factor to this strategy, it is also important to use specially adapted strains to biodegrade and/or endure the presence of xenobiotics (Essam et al. 2010).

In this perspective, the present study has aimed to characterize bacterial and microalgal strains in a local environment that degrades and tolerates one of the most commonly-used NSAID, ketoprofen, and the feasibility of development of microalgal/bacterial system for the biodegradation of pharmaceutical wastewater.

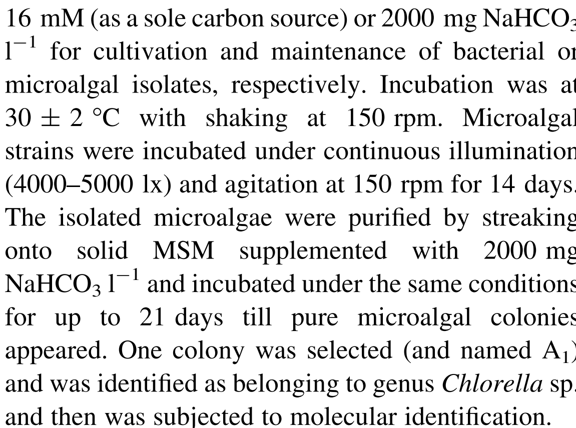
Materials and methods

All chemicals were reagent grade. Ketoprofen was obtained from Sigmatec Company for Pharmaceutical Industries, Egypt. Experiments under illumination were performed using LED lamps (12 W, 6500 K, 220240 V, 50/60 Hz). Unless otherwise specified, all experiments were conducted in triplicate at 30 ± 2 °C under sterile conditions. Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

Enrichment, isolation and maintenance of bacterial and algal strains

Biodegrading bacterial strains were isolated from wastewater samples collected from several sewage outlets of dyes or chemicals factories and areas near agricultural canals, also sludge samples were collected from a local sewage treatment plant. Microalgal strains were isolated from soil samples collected from different locations in Cairo.

Microbial isolation was carried out according to Essam et al. (2010) using ketoprofen from 0.25 to



16 mM (as a sole carbon source) or 2000 mg $NaHCO_3$ l^{-1} for cultivation and maintenance of bacterial or microalgal isolates, respectively. Incubation was at 30 ± 2 °C with shaking at 150 rpm. Microalgal strains were incubated under continuous illumination (4000–5000 lx) and agitation at 150 rpm for 14 days. The isolated microalgae were purified by streaking onto solid MSM supplemented with 2000 mg $NaHCO_3$ l^{-1} and incubated under the same conditions for up to 21 days till pure microalgal colonies appeared. One colony was selected (and named A_1) and was identified as belonging to genus *Chlorella* sp. and then was subjected to molecular identification.

The blue green microalga, *Spirulina platensis*, was kindly provided from Dr Abo El-Khair Badawy, Algal Biotechnology Unit, National Research Center, Egypt. It was maintained in modified MSM supplemented with 16.8 g $NaHCO_3$ l^{-1} according to the modified Zarrouk's medium. All isolated microorganisms were preserved by lyophilization.

Molecular characterization of K_2 consortium and microalgal strain

Biodegrading bacterial isolates were subjected to morphological and molecular characterization according to the protocol used in Rakaiby et al. (2012) using two universal primers: F-5′ACG CGT CGA CAG AG T TTG ATC CTG GCT-3′ and R-5′GGA CTA CCA G GG TAT CTA AT-3′ and the GenBank database (NCBI, USA) was then used to search for 16S rRNA sequence similarities. The isolated microalga A_1 was then subjected to molecular identification using Internal Transcribed Spacer (ITS) sequence analysis using two universal primers: ITS-1F 5′-GGTGAACCTGA GGAAGGAT-3′ and ITS-4R 5′TCCTCCGCTTATT GATATGC-3′. A_1 genomic DNA was isolated according to EMNE method (Eukaryotic Microalgal Nucleic acids Extraction) according to Kim et al. (2012) with some modifications. The GenBank database (NCBI, USA) was then used to search for ITS sequence similarities.

Quantitative determination of ketoprofen

Ketoprofen was analyzed by HPLC using an Agilent Eclipse analytical column (XDB-C18) with acetonitrile/50 mM potassium phosphate buffer pH 4, (3:2 v/v) at 2 ml min^{-1}. Detection was at 257 nm. Samples

from biodegradation experiments were filtered through a 0.45 μm filter and kept at −20 °C prior to analysis. Chemical oxygen demand (COD) was measured according to Essam et al. (2014).

Microalgal toxicity assay of ketoprofen

The assay was done according to the protocol used in (Essam et al. 2014) using two types of microalgae: the isolated green microalga; *Chlorella* sp. and the cyanobacterium *S. platensis.*

Brine shrimp lethality assay (BSLA) of ketoprofen

The assay (BSLA) was used to test the toxicity of ketoprofen. The cysts of *Artemia* sp. were first allowed to hatch according to Persoone (1986); the assay was performed in 12-well microplates using the freshly hatched nauplii. Different concentrations of ketoprofen were prepared in artificial sea water (ASW) (Kester et al. 1967) and 10 nauplii in the instar II and III stages were transferred to each well containing 5 ml of the respective concentration of ketoprofen. ASW was used as control, and the microplates were incubated at (30 ± 2 °C) under illumination of about 1000 lx intensity for 24 h. The mortality of the nauplii was then observed and the % lethality was determined according to the equation:

$$\%\ \text{Lethality} = \frac{\text{No.of dead } Artemia \text{ nauplii} \times 100}{\text{Initial no. of } Artemia \text{ nauplii}}$$

The assay was used to test the detoxification efficiency of ketoprofen by the K_2 consortium, and to test the toxicity of the metabolites which may be produced during biodegradation. The toxicity of the MSM against the brine shrimp was tested and 1/3 MSM dilution was the least dilution causing zero lethality (data not shown). Samples were diluted 3, 5 and 10 times with ASW prior to testing and MSM subjected to the same dilution was used as control.

Biodegradation pattern of ketoprofen by K_2 consortium under dark or continuous illumination conditions

The isolated K_2 bacterial consortium was cultured for 48 h on MSM slants supplemented with 5 mM ketoprofen. The bacteria were then harvested and adjusted to an OD_{600} value of 0.125. 100 ml MSM supplemented with 5 mM of ketoprofen was inoculated with 5 % (v/v) of the prepared bacterial suspension. Negative controls were prepared to determine the abiotic removal of ketoprofen by the same steps but without bacteria. Flasks were incubated for 8 days at 30 ± 2 °C with shaking at 150 rpm under continuous illumination (4000–5000 lx). For testing the biodegradation under dark conditions; test and control flasks were wrapped with aluminum foil, while in case of biodegradation under illumination; flasks were left unwrapped. Samples, 3 ml, were periodically withdrawn every 8 h and subjected to OD measurement, ketoprofen concentration determination by HPLC and COD analysis.

Determination of the maximum biodegradation capacity of K_2 consortium

This was tested under dark conditions in 100 ml MSM supplemented with 5, 7, 8, 10, 12, 16 or 20 mM ketoprofen. The test conditions were conducted as mentioned above, samples (2 ml) were withdrawn on days 0–5, 7, 14 and 28 and subjected to analysis of OD and ketoprofen concentration determination by HPLC.

Mass spectrometry analysis of biodegradation intermediates

Samples from the biodegradation experiment under dark conditions were extracted three times with equal volumes of ethyl acetate then the combined extracts were evaporated to dryness. The dried extracts were mixed with 5 ml acetonitrile, then directly injected into the Ultra-pressure liquid chromatography/electrospray ionization/mass spectrum/mass spectrum system. The UPLC system was connected to a triple quadropole mass spectrometer (Waters 3100) operated in negative ion mode, and the ions were generated using ESI ion source. The metabolites were detected by scan analysis and recording product ion spectra, operation conditions were as follows: desolvation temperature 400 °C, source temperature 150 °C, capillary voltage 4000 V and cone voltage 25 V.

Biodegradation of ketoprofen by K_2 consortium and *Chlorella* sp. under diurnal cycle conditions (12 h light/12 h dark)

A 5 % (v/v) of K_2 suspension initially adjusted to OD_{600} of 0.125 and/or 5 % (v/v) of *Chlorella* sp. culture harvested in the rapid growth phase of growth adjusted initially to contain 10 mg chlorophyll (Table 1), were inoculated into serum bottles of 120 ml filled with 100 ml MSM and supplemented with 2 mM ketoprofen (500 µg ketoprofen ml^{-1}) as a sole carbon source. The bottles were then sealed with rubber septa and agitated at 150 rpm at 30 ± 2 °C under diurnal cycle conditions for 10 days. Samples were withdrawn on days 0–5, 7 and 10 for determination of OD, chlorophyll a content, ketoprofen concentration by HPLC and COD measurement. All sets were carried out in triplicates.

Results

Identification and characterization of the isolated bacteria and microalgal species

The K_2 bacterial consortium biodegraded ketoprofen as a sole carbon and energy sources. Four different colonies arose from this consortium on nutrient agar medium. They were molecularly identified as: *Raoultella ornithinolytica* B6 (Genbank accession number CP004142), *Pseudomonas aeruginosa* strain JPP (Genbank accession number KJ626420), *Pseudomonas* sp. P16 (Genbank accession number EF628000) and *Stenotrophomonas* sp. 5LF 19TDLC (Genbank accession number FN666617). The isolated microalga A_1 strain was molecularly identified to be *Chlorella* sp. Iso4 (Genbank accession number (JX041600).

Toxicity assays of ketoprofen

The isolated green microalga *Chlorella* sp. was more resistant to ketoprofen than *S. platensis* cyanobacterium, and *Artemia* sp. (Fig. 1).

Biodegradation pattern of ketoprofen by K_2 consortium under dark or continuous illumination conditions

K_2 consortium degraded up to 16 mM ketoprofen in the dark, the biodegradation became slower as the concentration increased. Under dark conditions, the rate of biodegradation of 5 mM ketoprofen was 33 µg ketoprofen h^{-1}, complete biodegradation was achieved within 48 h, with a lag phase of about 10 h, while the control flasks did not exhibit any degree of abiotic degradation. Ketoprofen was not sensitive to visible light (Fig. 2). However, the biodegradation of ketoprofen was hindered by light where the biodegradation rate decreased to 7.6 µg ketoprofen h^{-1} with a prolonged lag phase to 72 h. A statistically significant difference between the biodegradation rate of ketoprofen under dark or continuous illumination conditions was observed. [The analysis was performed using Student's *t* test, the difference is considered significant if p value <0.05[1]]. In case of biodegradation under darkness, almost 41 % COD was removed within 8 days. Application of continuous illumination conditions showed no remarkable reduction in COD values before day 5, and then 40 % COD removal was observed on day 7. Under both darkness and illumination, no COD removal was observed in the negative control flasks (Fig. 3). Samples from the biodegradation experiments were diluted 3, 5 and 10 times with ASW prior to BSLA. The results of the lethality assay

Table 1 Experimental design for the treatment of artificial wastewater supplied with microalga *Chlorella* sp. (A), bacterial consortium K_2 (B), diurnal cycle illumination pattern (D) and ketoprofen (K), where KD = MSM supplemented with ketoprofen under diurnal cycle conditions, BKD = MSM supplemented with ketoprofen + K_2 bacterial consortium under diurnal cycle conditions, AKD = MSM supplemented with ketoprofen + the algal strain (*Chlorella* sp. Iso4) under diurnal cycle conditions and ABKD = MSM supplemented with ketoprofen + K_2 bacterial consortium + the algal strain (*Chlorella* sp. Iso4) under diurnal cycle conditions

Test	KD	BKD	AKD	ABKD
A	–	–	+	+
B	–	+	–	+
K	+	+	+	+
D	+	+	+	+

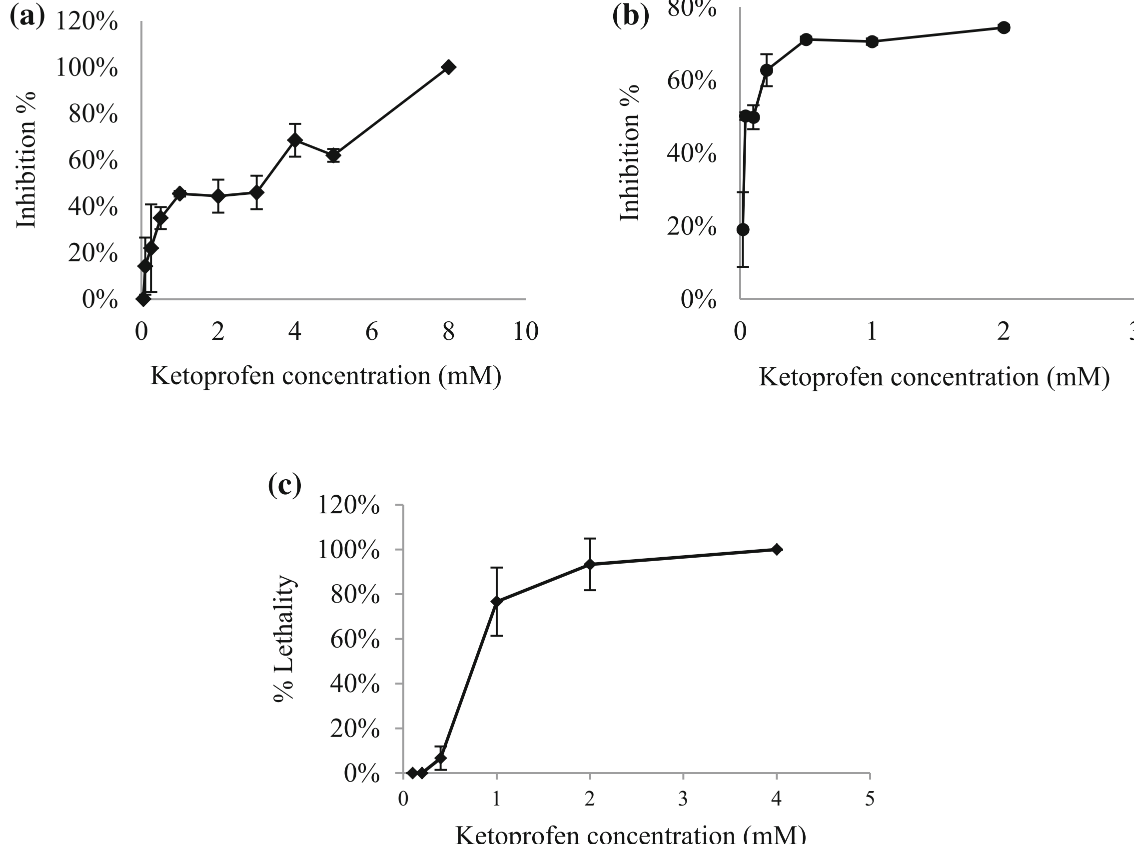


Fig. 1 Percentage growth inhibition of *Chlorella* sp. Iso4 **(a)** and *S. platensis* **(b)** represented by reduction in the chlorophyll a content after incubation in MSM supplemented with different ketoprofen concentrations for 72 h at 30 ± 2 °C under continuous illumination (4000–5000 lx) and agitation (150 rpm), **c** lethality percentage of the brine shrimp at 30 ± 2 °C under continuous illumination (~1000 lx) for 24 h for brine shrimp by ketoprofen

using the ten-fold diluted samples showed that the toxicity of ketoprofen was reduced from 17 % to only 3 % after biodegradation under either dark or continuous illumination conditions.

Mass spectrometry analysis of biodegradation intermediates

The analysis of samples from ketoprofen biodegradation under dark conditions using mass spectrum scan revealed that ketoprofen was degraded to three main metabolites (Fig. 4), Initially (time zero), a major peak observed with *m/z* 253 was that corresponds to the parent drug ketoprofen. The first detected peak was observed at *m/z* 209 after 12 and 24 h. A peak with *m/z* 197 appeared after 24 h, finally at 48 h and 72 h samples a peak with *m/z* 165 appeared (Table 2).

Biodegradation of ketoprofen by K_2 bacterial consortium and *Chlorella* sp. under diurnal cycle

Four sets were prepared:

1. MSM supplemented with ketoprofen under diurnal cycle conditions (KD).
2. MSM supplemented with ketoprofen + K2 bacterial consortium under diurnal cycle conditions (BKD).
3. MSM supplemented with ketoprofen + the algal strain (*Chlorella* sp. Iso4) under diurnal cycle conditions (AKD).

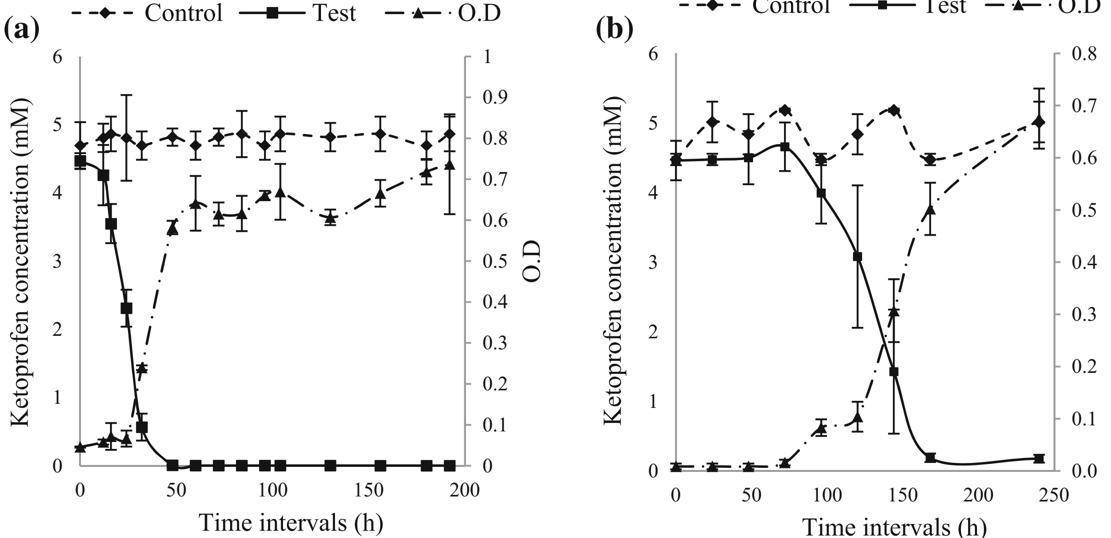


Fig. 2 Biodegradation pattern of 5 mM ketoprofen by K_2 consortium correlated to bacterial biomass produced under dark conditions **a**, under continuous illumination conditions with LED lamps, at light intensity of 4000–5000 lx **(b)**

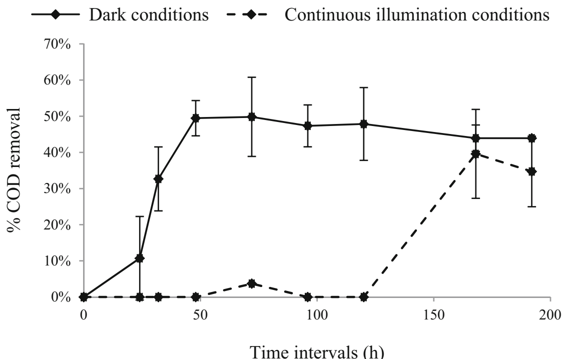


Fig. 3 The % COD removal values during biodegradation of ketoprofen by K_2 consortium under either dark or continuous illumination conditions

4. MSM supplemented with ketoprofen + K2 bacterial consortium + the algal strain (*Chlorella* sp.208 sp. Iso4) under diurnal cycle conditions (ABKD).

Neither KD nor AKD nor BKD showed biodegradation or considerable COD removal (Fig. 5). Only in case of ABKD set, ketoprofen was degraded at 3.5 µg ketoprofen h^{-1} within 10 days after a lag phase of about 24 h. Initially the ABKD and AKD sets contained 0.44 mg chlorophyll a l^{-1}, then in ABKD set only it started to increase starting from the 2nd day and reached about 8.5 mg chlorophyll a l^{-1} on day 7 then became constant (while in AKD set, no increase in the chlorophyll a content was obtained). Also there was 50 % COD removal on day 5 in this set which only became constant at the end of the experiment. When the biodegradation rate in ABKD set was compared with other sets, a statistically significant difference was observed (Student's *t*-test, *p*-value <0.05).

Discussion

Consortia of bacteria can biodegrade a wide range of aromatic compounds, such as phenols and related compounds (Rakaiby et al.2012; Wojcieszyńska et al. 2014). The greater resistance of *Chlorella* sp. to ketoprofen than *S. platensis* is in agreement with (Tastan et al. 2012) who found that *Chlorella* sp. was more tolerant than *Synechococcus* sp. to acidic pollutants.

The more rapid biodegradation rate of ketoprofen in the dark than under continuous illumination may be attributed to the reported phototoxicity of ketoprofen with the production of photoproducts without a rapid deactivation mechanism, this may inhibited the biodegradation activity of K_2 consortium and so more time was needed for the recovery of this activity (Durbize et al. 2003). The 82 % reduction in the lethality of the tested nauplii after biodegradation

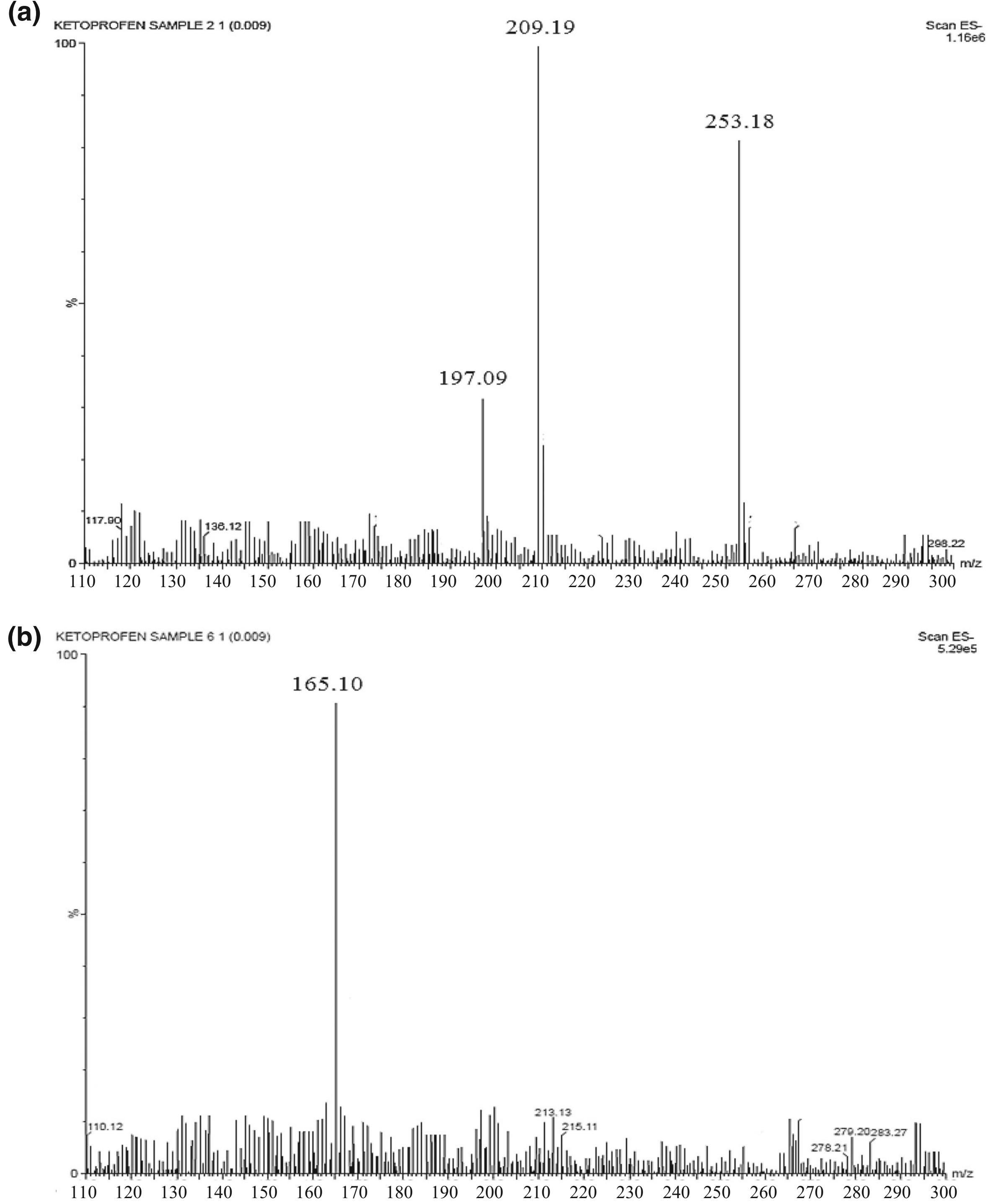


Fig. 4 **a**, **b** Chromatograms of MS/MS analysis of possible intermediates detected during ketoprofen biodegradation

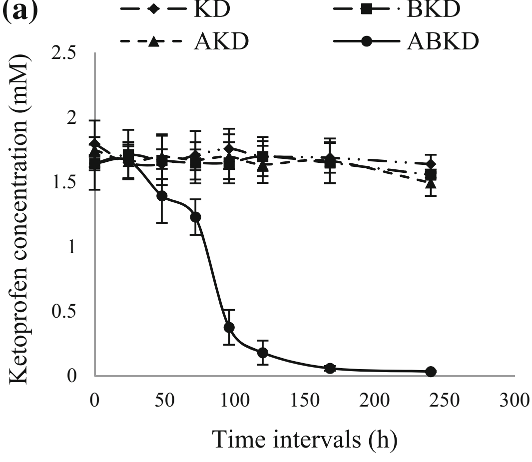
Table 2 Molecular anions and suggested metabolites determined during the biodegradation experiments

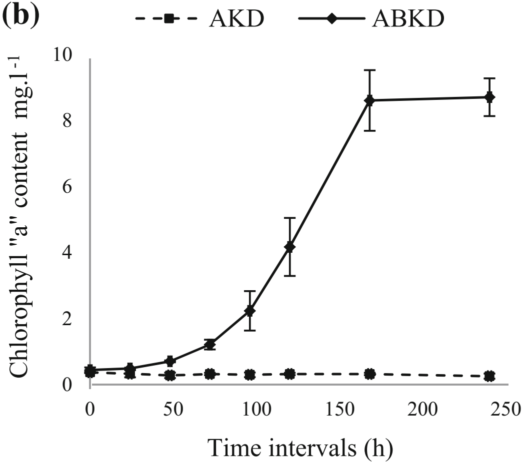
Molecular anion (*m/z*)	Suggested metabolite	Structure of the suggested metabolite	Time interval of sample
253	Ketoprofen (parent)		Zero time
209	(3-ethylphenyl)(phenyl)methanone		12 and 24 h
197	(3-hydroxyphenyl)(phenyl)methanone		24 h
165	(3-hydroxyphenyl)(oxo)acetic acid		48 and 72 h

suggests that K_2 consortium detoxified ketoprofen to less harmful compounds. MS/MS analysis indicated that the first step is the decarboxylation of ketoprofen, followed by series of oxidation/reduction reactions until complete removal. These results are not in agreement with Quintana et al. (2005) and Marco-Urrea et al. (2010b) The former has proposed that ketoprofen was partially mineralized using an activated sludge system and that the degradation followed the pathway for degradation of biphenyls and related compounds, while Marco-Urrea et al. (2010b) reported that three intermediates were identified with complete mineralization of ketoprofen by the white-rot fungus *Trametes versicolor*. As the masses of the metabolites detected in this study do not match those of the reported ones, this suggests a novel pathway for ketoprofen biodegradation.

Chlorella sp. was chosen to be combined with the K_2 consortium for ketoprofen biodegradation due to its higher resistance than *S. platensis*. A diurnal cycle was chosen to shorten the lag phase which observed under continuous illumination conditions. No degradation of ketoprofen occurred in KD, BKD or AKD sets. This indicated that visible light emitted by the LED lamps did not degrade the drug and that *Chlorella* sp. did not degrade ketoprofen. However; many studies have reported that *Chlorella* sp. can degrade some toxic organic aromatic compounds such as 2,4-dimethyl phenol, 2-chlorophenol and bisphenol A (Peng et al. 2006); while others have reported this genus can only tolerate organic pollutants (Rakaiby et al. 2012). Also the low availability of O_2 in the sealed serum bottles with a small headspace left in the BKD set may be the reason for absence of ketoprofen biodegradation. A similar observation was recorded in other studies (Tamer et al. 2006).

The observed increase in chlorophyll a content in the ABKD set can be explained in terms of biodegradation pattern where the biodegradation started after a lag phase of about 24 h when ketoprofen was degraded with concomitant production of CO_2 utilized by *Chlorella* sp. leading to continuous increase in the biomass as a result of photosynthesis till complete removal of ketoprofen was achieved. This was confirmed by the significant reduction in COD values by about 50 %.





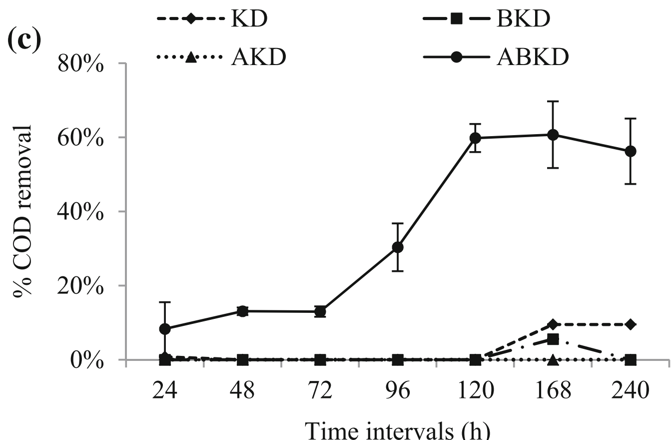


Fig. 5 Biodegradation pattern of ketoprofen using microalgal–bacterial consortium within 10 days **(a)**, the measured chlorophyll a content of AKD and ABKD sets for 10 days **(b)**, COD removal % for each set of ABKD experiment **(b)**, where KD = MSM supplemented with ketoprofen under diurnal cycle conditions, BKD = MSM supplemented with ketoprofen + K_2 bacterial consortium under diurnal cycle conditions, AKD = MSM supplemented with ketoprofen + the algal strain (*Chlorella* sp. Iso4) under diurnal cycle conditions and ABKD = MSM supplemented with ketoprofen + K_2 bacterial consortium + the algal strain (*Chlorella* sp. Iso4) under diurnal cycle conditions

Conclusion

The combined isolated bacterial and microalgal strains had a high efficiency for ketoprofen biodegradation and tolerance. These isolates are good candidates for cost effective bioremediation of heavily-loaded wastewater effluents from pharmaceutical industries.

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