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Removal of metronidazole from aqueous media by *C. vulgaris*

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Highlights

1. Metronidazole is efficiently removed during algal treatment.
2. Metronidazole is removed via sorption onto the biomass.
3. Metronidazole showed toxicity to *C. vulgaris* at stationary phase of life cycle.
4. The removal efficiency of *C. vulgaris* decreased with increase of antibiotic.

Abstract

This current study investigated the removal of metronidazole from aqueous media by *C. vulgaris*. Two different initial sizes of inoculum (0.05 and 0.1mg/L) were tested for a wide concentration range of metronidazole (1-50µM). The effect of metronidazole concentrations on biomass production was studied for 20 days. The exopolymeric substances (EPS) were quantified and correlated with the removal of antibiotics from aqueous media. Specifically, MDZ stimulated the production of EPS in *C. vulgaris*, which played the major role in the adsorption of this antibiotic. Also, metronidazole significantly influenced the zeta potential of *C. vulgaris* in the test cultures, indicating a change in surface characteristics. This decrease in surface negative charge caused auto-flocculation phenomena at a stationary phase. Chronic and acute toxicity experiments showed that metronidazole was harmful to *C. vulgaris* at stationary phase. Results from this study would advance our knowledge on the treatment of metronidazole-contaminated waters with *C. vulgaris* as a green technology-oriented process.

Keywords: Adsorption; *C. vulgaris*; EC50; metronidazole

1. Introduction

The excessive use of pharmaceuticals and their improper disposal pose risks to the environment and public health [1]. Especially, antibiotics (i.e., widely used for preventing bacterial diseases in humans and livestock) are continuously introduced in natural aquatic environments [2, 3]. Despite their low concentrations, antibiotics potentially affect the quality of drinking water, human health, and ecosystems [4]. The residual antibiotics occurring in aquatic environments would lead to the emergence of resistance among targeted and non-targeted strains of microorganisms [5-7]. Also, surface waters contaminated with antibiotics expand the variety of resistant microorganisms and the transfer of genetic material between multidrug-resistant bacteria [8, 9].

Metronidazole is a first-generation derivative of 5-nitroimidazole drugs developed for therapeutic uses [10, 11]. Metronidazole is an effective prescription medication used for treating several infectious diseases caused by anaerobic bacteria and protozoa in humans and livestock [12, 13], showing minimal side effects and a broad spectrum of applications [14]. In general, antibiotics have a relatively short half-life in open environments (e.g., water bodies or soils) [2]; however, metronidazole is highly persistent in nature due to its high solubility [15] and low biodegradability [16]. Also, the removal of metronidazole from water and wastewater during the treatment train is not efficient [17]. Deblonde et al., [18], reported a mere 38.9% removal of metronidazole from a wastewater treatment plant. Nevertheless, because of its high toxicity and mutagenic effect on targeted and non-targeted microorganisms in the environment [12], it is highly necessary to develop efficient protocols and (bio) technologies to remove this antibiotic from aqueous media.

Several methods have been reported for the removal of metronidazole from aqueous media. Briefly, chemical processes such as reduction [19], Fenton and photo-Fenton process [20], UV-photolysis [21], and physical treatments (e.g., adsorption) [22, 23] have been mainly tested. However, high energy and chemical consumptions, as well as the generation of secondary waste are the main constraints for their successful (economic) implementation [24, 25]. On the other hand, Rivera-Utrilla et al., [22] had reported that microorganisms in wastewater treatment plants cannot efficiently degrade metronidazole. Activated sludge process is the most common biological treatment method to remove most of the drugs from aqueous media; however, this process fails to efficiently remove most of the antibiotics [26]. In general, antibiotics are not readily biodegradable [27]. Antibiotics are mainly removed by sorption onto activated sludges in wastewater treatment plants [4, 28]. However, antibiotics desorb from the activated sludge to the aqueous media during its reuse in aeration tanks to maintain a fixed concentration of activated microorganisms [29]. According to Grandclement et al., [30] wastewater treatment plants using activated sludge are not specifically designed to remove antibiotics.

Simultaneously, the activated sludge in wastewater treatment plants promotes the occurrence of antibiotic-resistant bacteria due to the exposure of bacteria to antibiotics present in wastewater [31]. Therefore, the efficient treatment of antibiotics present in wastewater to ensure biosafety for humans and their environment is a difficult task [32].

The use of microalgae as green technology is a viable option to remove antibiotics from water. Moreover, microalgal cultures mitigate atmospheric CO_2 while the biomass can be used for biofuel feedstocks. Briefly, microalgae are known for efficiently removing xenobiotic compounds from water, including a range of pharmaceuticals. Garcia-Rodríguez et al., [33] studied the role of microalga *Spyrogira* sp. in photolysis degradation and the contribution of light

in the total removal of six antibiotics from the sulfonamides and tetracyclines families. Du et al., [34] investigated the response of green algae *Chlorella pyrenoidosa* towards the removal of ceftazidime antibiotic. Guo and Chen [2] showed the removal of cephalosporins from wastewater using *Chlorella pyrenoidosa*. Conversely, Magalhães et al., [16] unsuccessfully attempted to remove metronidazole from water using *Microcystis protocystis* (cyanobacteria). From these previous studies, chlorophytes have clearly shown more resistant to stress conditions during water treatment. *Chlorella sp.* is a chlorophyte which has been extensively used for the removal of antibiotics such as 7-amino cephalosporanic acid [32], cephalosporins [2], norfloxacin [35], levofloxacin [36], and enrofloxacin [37]. However, to the best of our knowledge, metronidazole removal using *C. vulgaris* has not yet been reported.

This current study has investigated the metronidazole removal capacity of *C. vulgaris* and the dominant mechanisms involved in the removal. The impact of metronidazole at different concentrations on the growth rate of *C. vulgaris* was examined. Additionally, the tolerance and chronic toxicity (EC50) of *C. vulgaris* to this antibiotic as a function of its stage of life cycle was investigated. Finally, the exo-polymeric substances (EPS) production by *C. vulgaris* during exposure to metronidazole, its influence on the surface properties of *C. vulgaris,* and its role during the removal of this antibiotic were comprehensively studied. Results from this study will advance our knowledge of the treatment of metronidazole-contaminated waters with *C. vulgaris*. The protocols followed in the current investigation can be extended to the treatment of different antibiotics-contaminated aquifers using green technology-oriented processes. Simultaneously, a dual-approach can be used in the system to treat wastewater and to produce valuable biomass as feedstock for biofuel, through anaerobic digestion. This latter process would aim to completely degrade any antibiotic residue [61].

2. Materials and Methods

2.1. Chemical reagents

Metronidazole (HPLC grade; >99% purity) was obtained from Sigma-Aldrich. All the chemicals, solvents, and reagents used were of analytical grade. A stock solution of 1mM metronidazole (MDZ) was prepared with 30% methanol and 70% MilliQ water (v/v) and stored in a dark airtight glass vial at 4ºC for no longer than 30 days for further experiments.

2.2. Algae Culturing and batch experiments

Chlorella vulgaris (*C. vulgaris*) was obtained from CSIRO Marine Research Hobart, Tasmania. *C. vulgaris* was re-cultured in 2 L conical flasks with a working volume of 1L in BG11 media (Table 1), at 25±4°C. The cultures were exposed to fluorescent lamps to provide the irradiance of 120 µmol photon /m^2/s until the stationary phase was attained.

Two sets of batch experiments were conducted. The freshly prepared culture media were inoculated with two different sizes of inoculum of *C. vulgaris* for obtaining 0.05 g/L and 0.5 g L^{-1} biomass (i.e., dry biomass vs optical density calibration curve) in 3 L of Erlenmeyer flasks with 2 L of working volume. The cultures were spiked with increasing concentration of MDZ (1-50 µM) except for the control cultures of each batch. All experiments were conducted in triplicate.

2.3. Determination of biomass and chlorophyll-a

The determination of the biomass of the test and control cultures was conducted every day by measuring the optical density at 680 nm as described in [38]. A batch study was conducted to evaluate the specific growth rate using the following formula;

$$\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1)$$

Where, N_1 and N_2 are biomass concentrations (mg L^{-1}) at day t_1 and t_2, respectively, during the exponential phase of growth. All experiments were conducted in triplicate.

The extraction of chlorophyll-a and its quantification was conducted as reported by Xiong et al., [39]. A 10 mL of *C. vulgaris* was centrifuged at 4500 rpm for 10 min. The supernatant was discarded and the pellet was collected. The pellet was re-suspended in 10 mL of methanol:water (9:1; v/v) and incubated in 20 mL centrifuge tubes for 10 min. The incubated culture was centrifuged again at 4500 rpm for 10 min. The absorbance of the supernatant was measured at 665 nm and 652 nm and the chlorophyll-a concentration of the extract was calculated using the following equation:

$$\text{Chlorophyll-a } (\mu g\ L^{-1}) = 16.29 \times A_{665} - 8.54 \times A_{652}$$

Where, A_{665} and A_{652} are the absorbance of the supernatant at wavelength 665 nm and 652 nm, respectively.

2.4. Toxicity of MDZ for C. vulgaris

The toxicity of MDZ was calculated as the Effective Concentration (EC50) of the stressor (MDZ) at which 50% of the growth rate of *C. vulgaris* was inhibited. The inhibition in growth was calculated as a function of chlorophyll-a content. To determine the chronic toxicity of MDZ, a batch experiment was conducted at different concentrations of MDZ (65-90 µM) with an initial biomass concentration of 0.1 g L^{-1}. The microalgae were exposed to antibiotic for 240 hours (10 days). However, for acute toxicity experiments, a 0.1 g L^{-1} culture at stationary phase (20 days old culture) was spiked with 1-50 µM antibiotics for 96 hours. The growth rate was calculated in terms of chlorophyll-a (µg L^{-1}).

161 *2.5. Extraction and analysis of Exo-polymeric substances (EPS)*

162 Samples of 50 mL of *C. vulgaris* cultures at late exponential phase (i.e., which started
163 approximately from day 10, Fig 1) were kept in the fridge at 4°C to settle the cells. The
164 supernatants were decanted and used for EPS analysis, while the settled cells were re-suspended
165 in 50 mL of MilliQ water in a beaker. The culture was stirred for 2 hours at 1000 rpm using a
166 magnetic stirrer to extract the EPS from the cell wall of *C. vulgaris.* Then, the cells were
167 separated by centrifugation at 1000 rpm for 10 minutes. The second supernatants were collected
168 for analysis of bound EPS. The pellets of the centrifuged biomass were dried at 60°C. The EPS
169 were extracted at every alternate day starting from day 10 until day 20.

170 30 mL of all supernatants were used for measuring the dry weight of EPS. Every supernatant of
171 the tests and control cultures of age 10 days and beyond were used for EPS determination. 30 mL
172 of supernatants were dried in an oven at 60°C until a stable weight was achieved to calculate the
173 ratio of the dry weight of EPS to biomass.

174

175 *2.6. Zeta potential*

176 The Zeta (ζ) potentials of *C. vulgaris* from the test (low and high inoculum spiked with 5 µM
177 MDZ) and control cultures were analyzed on every alternate day from day 0 till day 20 using a
178 Zetasizer (Malvern). 10mL of cultures were collected from the Erlenmeyer flasks. The biomass
179 was allowed to settle down. The supernatants were discarded and biomass was re-suspended in
180 MilliQ water. The suspended *C. vulgaris* was pipetted in a folded cuvette. The zeta potential was
181 measured 5 times and the average and standard deviation were calculated.

182

183 *2.7. Analysis of MDZ*

184 Samples of 5 mL from test bioreactors were collected and filtered by a syringe filter (0.45 µm)
185 and analyzed using an HPLC equipped with UV/VIS detector (Agilent 1100 series, USA) at 320
186 nm. A 250 mm*4.6 mm C18 5-µm reverse phase HPLC column (Alltima^{TM}, GRACE) was used
187 and the mobile phase consisted of 0.1% phosphoric acid solution and methanol (80:20 v/v) with
188 a 1 mL min^{-1} flow rate. To analyze the removal mechanism and removal efficiency of MDZ via
189 *C. vulgaris*, flocculated biomasses of microalgae were collected from the bottom of a bioreactor
190 (i.e., a random selection was conducted from a 10 µM MDZ high biomass bioreactor on day 20)
191 in triplicate. The biomasses were washed thrice with 5 mL MilliQ to remove the unbounded
192 molecules of MDZ from biomass using filter paper (GF/C; 1.2 µm) and the filtrates were
193 collected in HPLC vial for analysis after every wash. The absence of MDZ peak after third wash
194 approved the adequacy of three washes of collected biomasses for further steps of analysis. The
195 washed biomasses were collected on glass Petri dishes separately and vacuum dried at 60°C in
196 temp controlled oven for 24 hours till they achieved constant weight. 0.5 mg of dried biomass
197 was suspended into 20 mL of ethyl acetate followed by sonication for 15 minutes and
198 centrifuging at 12,000 rpm for 10 minutes [16]. The biomass was separated from ethyl acetate
199 using a syringe filter (GF/C; 1.2 µm). The filtrate was vacuum dried at 40°C, followed by
200 diluting the remaining residue in 20 mL fresh culture media. The final diluted residue (i.e.,
201 expecting desorbed MDZ in solution) was filtered with a 1.2 µm GF/C syringe filter and
202 collected in HPLC vial for analysis. The results were used to elucidate the MDZ removal
203 efficiency of *C. vulgaris* and the mechanism involved during the removal process.
204

205 **3. Results and discussion:**

206 3.1. *Effects of MDZ on the growth of* C. vulgaris:

207 Two sizes of inoculum (0.05 and 0.5 g L^{-1}) were selected to study the effect of MDZ on *C.*
208 *vulgaris* concentration in solution. These results were compared with those of control culture. As
209 depicted in Fig 1a-b (Refer to Table S1a-b for standard deviations), the growth of the cultures
210 can be divided into three phases. The early exponential phase was calculated for 10 days from
211 the beginning of the culture due to the highest growth rate during this period for all tests and
212 control culture regardless of inoculum size. Interestingly, the specific growth rates of *C. vulgaris*
213 during the early exponential phase were almost similar for cultures with the same inoculum size
214 but different concentrations of MDZ (Table 2). Clearly, there was no impact of MDZ on *C.*
215 *vulgaris* during this early exponential phase. Remarkably, there was no removal of MDZ from
216 any of the test bioreactors during the early exponential phase (Figure 2a-b, Table S2a-b). During
217 the late exponential phase (following the first 10 days of growth), the biomass increased with
218 similar specific growth rate for the same inoculum size. These results indicate that MDZ does not
219 impact the specific growth rate of the culture. However, MDZ hampered the duration of the late
220 exponential growth phase, where MDZ decreased the surface charge of the cells causing them to
221 flocculate and settling down (Fig 3).
222 The duration of the late exponential phases was different in different test bioreactors. The longest
223 duration was observed for control cultures as 8 days; nevertheless, the control culture of high
224 inoculum attained a lower specific growth rate than control culture and low inoculum (Table 2).
225 The test cultures with low inoculum remained in late exponential phase for 5-6 days, while high
226 inoculum test cultures showed the shortest duration of 2-3 days. The longer late exponential
227 phase for low inoculum cultures might be due to: 1) a lower concentration of microalgal cells
228 present in the media than higher inoculum bioreactors for the same amount of nutrients, and 2)
229 late auto-flocculation in low inoculum cultures. The late auto-flocculation in low inoculum test

230 bioreactors caused biomass to remain in suspension media for a longer time and hence increased
231 the duration of the late exponential phase. The concentrations of MDZ start decreasing during
232 late exponential phase in all cases regardless of inoculum sizes (0.05 and 0.5 g L^{-1}) or initial
233 concentrations (1-50 µM) of MDZ as shown in Fig 2a-b. These results confirmed that MDZ
234 played a major role in auto-flocculation as further explained in next section. In all test
235 bioreactors, the stationary phase did not last for more than 24 hours, which was then followed by
236 a quick decline in biomass content. The reason for this rapid decrease in suspended biomass was
237 due to auto-flocculation; however, the removal of MDZ continued even during the decline in
238 biomass phase.
239
240 3.2. *Effect of MDZ on zeta potential of* C. vulgaris:
241 Fig 3 revealed the electronegative zeta potential of *C. vulgaris* from control culture and test
242 bioreactor spiked with 5 µM MDZ (i.e., which was randomly selected as a representative case).
243 In both cases, the electronegative zeta potential decreased with the age of the culture.
244 Throughout the growth cycle of the microalgae, the concentration of the functional groups
245 present on the cell membrane and cell wall changes and influences the surface properties of the
246 cells [40]. However, during the active growth phase the lower intercellular interaction caused a
247 higher net electronegative zeta potential [41]. At late exponential growth phase, the metabolism
248 of the cells and the mobility decreased. Simultaneously, the changes on the cell surface caused
249 intercellular interactions which resulted in a lower electronegative zeta potential [41]. Xia et al.,
250 [42] reported that the decrease in the electronegative zeta potential of the *C. vulgaris* was also
251 due to the increase in the effective (hydrodynamic) size of the cells (i.e., cell with bound EPS) at

252 a later stage of its life cycle. Decho and Gutierrez [43] considered EPS as an extended part of the
253 cell.
254 Fig 3 showed that the electronegative zeta potential of the *C. vulgaris* decreased more
255 significantly in the test bioreactors than their respective control cultures. Among the test
256 bioreactors, the cultures with low inoculum size showed a higher electronegative zeta potential
257 than the high inoculum size culture at day 12 and beyond. The high inoculum culture contained
258 more biomass, which attained a stationary phase earlier than low inoculum cultures. Since the
259 EPS forms at late exponential or stationary phase of life cycle [43], high inoculum cultures start
260 forming EPS earlier than the low inoculum culture (Table 3).
261 As mentioned earlier, the net negative zeta potential of the microalgal cell decreased due to
262 change in functional groups present on the cell wall, which would be directly associated with the
263 metabolic conditions of the cells [40]. However, in the current study, the additional decrease in
264 zeta potential compared to their control cultures could be due to the charge dispersion interaction
265 of MDZ with algal cells (discussed in section 3.4) as well as the increase in the effective size of
266 the cell. The effective size of the cells would be larger in test cultures compared to their
267 respective control cultures due to the formation of bound EPS onto the cells in test cultures
268 (Table 3).
269 A drop in zeta potential in the test cultures indicated a decrease in the repulsive electrostatic
270 forces between the cells which led to auto-flocculation. The auto-flocculation takes place when
271 van der Waals forces overcome the repulsive electrostatic forces [44]. In this study, the repulsive
272 electrostatic forces were most likely decreased due to the adsorption of EPS around the cells and
273 also because of its interaction with MDZ in its scaffolds.
274

3.3. *Effect of MDZ on the EPS production of* C. vulgaris

EPS is produced by microalgae at a later stage of life cycle [43]. Microalgae produce EPS for cell protection as a waste of metabolic processes [45]. There are two types of EPS produced by *C. vulgaris*; a) EPS soluble (free) in media, and b) loosely bound (adsorbed) EPS which assists in auto-flocculation of cells [46]. EPS is generally discussed as a three-dimensional architecture scaffold providing space for trapping foreign compounds and slowing the diffusion rate, physical stability of cells, digestion of xenobiotic substances, and communication between cells (i.e., quorum sensing) [43].

Control culture produced more soluble (free) EPS than bound (adsorbed) EPS (Table 3). This result explains why control culture cells did not undergo auto-flocculation. However, in test bioreactors the auto-flocculation phenomenon was common. A slight increase in bound EPS was observed with an increase of MDZ concentration in both test cultures (low and high inoculum). The bioreactor spiked with 1 µM MDZ produced significantly less EPS (47.7 ± 1.23 mg g^{-1} DW of *C. vulgaris*) than that with 5 µM (151.6 ±3.45 mg g^{-1} DW of *C. vulgaris*), which indicated that MDZ stimulated the EPS production in *C. vulgaris*. However, concentrations higher than 5 µM MDZ produced almost the same amount of EPS, which varied from 151.6-145.5 mg g^{-1} dry weight (DW) and 139.3-146.1 mg g^{-1} DW for low and high biomass bioreactors, respectively. These results revealed that 5 µM MDZ was the optimum concentration of antibiotics to produce maximum EPS for this specific system.

3.4. *Removal of MDZ*

MDZ was analyzed from day 0 until day 20 in test bioreactors. The removal of MDZ by *C. vulgaris* from aqueous media under different conditions is shown in Figure 2a-b. MDZ was not

298 removed until the early exponential growth phase. This result proved that MDZ did not adsorb
299 onto the cell surface of *C. vulgaris* possibly due to the low log K_{ow} (-2.3) value of MDZ. Also,
300 the pH of the *C. vulgaris* culture varied between 9-10, where MDZ is expected to behave as a
301 neutral molecule [47]. Therefore, electrostatic interactions between MDZ and negatively-charged
302 *C. vulgaris* would not be expected. However, π-electron of the heterocyclic aromatic ring of the
303 MDZ molecule would interact with different sites of EPS (Figure 4). EPS are highly hydrated
304 complex polymers mainly composed of polysaccharides, proteins, and DNA [48]. The CH group
305 of the polysaccharides form CH-π bond with the π-electrons of aromatic compounds [49, 50].
306 Consequently, the adsorption of MDZ molecules in the scaffold of the EPS matrix would involve
307 van der Waals interaction between π-electron of the heterocyclic aromatic ring of the MDZ
308 molecule and the hydrogen atom of CH group of the polysaccharides to form a CH-π bond.
309 Another possible mechanism would involve OH-π bond with OH groups of saccharides.
310 However, EPS has a complex structure where the OH groups of polysaccharides involved in
311 intermolecular interactions, might have no free OH to make bonds with MDZ [49]. Nishio [51]
312 has reported that saccharides are overall polar in nature but include some hydrophobic moieties
313 depending upon the orientation of OH groups, which have an affinity to form hydrogen bonds
314 with hydrophobic compounds [49]. The hydrophobic imidazole ring in MDZ would form OH-π
315 bond with OH groups of hydrophobic moieties. Previous studies have reported that MDZ
316 interacts with its π electron of heterocyclic aromatic ring to form π-π dispersion bonds with other
317 aromatic compounds [22]. Protein containing aromatic amino acids would be expected to favor
318 the formation of π-π dispersion bonds for MDZ to interact with EPS. Sanvordeker et al., [52]
319 confirmed that the alkyl side chain of MDZ participates in the binding to proteins. Besides, EPS

320 contains a diverse range of functional groups, including amine, carboxylate, phosphate, carbonyl,
321 amide, and hydroxyl which can form H-bonds with MDZ.
322 The removal of MDZ started after day 10 in all cases regardless of MDZ or biomass
323 concentration. Remarkably, MDZ removal and EPS formation occurred almost simultaneously.
324 These results indicated that the MDZ might be removed by EPS solely by adsorption
325 mechanism. Neither metabolites nor antimicrobial biotransformation products were observed in
326 HPLC chromatogram; suggesting that the EPS of *C. vulgaris* might not contain any metabolizing
327 enzyme for MDZ. Therefore, it is postulated that the decrease in MDZ concentration in the
328 bioreactors would only be associated with adsorption phenomena, which eventually played a role
329 in increasing the concentration of EPS. As a control experiment, bound EPS was extracted from
330 10 µM high biomass bioreactor, sonicated for 15 minutes, followed by filtration and analysis,
331 where the presence of MDZ was clearly recorded. A bioreactor has a complex system of
332 suspended and flocculated biomass; thus, achieving an accurate mass balance of MDZ was not
333 possible. However, these results showed that 0.69 mg MDZ adsorbed per 100 mg of dry weight
334 of EPS (i.e., corresponding to 59% of initial DMZ) in a 10 µM MDZ-high biomass bioreactor
335 after 20 days of incubation. The small molecular size of MDZ would favor its adsorption into the
336 scaffold of EPS, while the concentration gradient would play a major role in the diffusion of
337 MDZ into EPS [53].
338 A comparison between the MDZ removal of low and high biomass-containing bioreactor
339 revealed that the longer the late exponential growth phase, the lower the removal efficiency.
340 Specifically, a longer late exponential growth phase delayed the EPS production. In the case of 5
341 µM MDZ bioreactors, the low biomass reactor was in active phase until day 15, while the active
342 or growth phase was over on day 12 in the high biomass reactor. Taking into account both

343 biomass and EPS, it was clear that EPS initiated the adsorption; however, the higher the biomass,
344 the better was the removal.

345 *3.5.Effect of initial concentrations of MDZ in culture media*

346 Wastewaters from different geographic locations contain different concentrations of
347 pharmaceuticals. Consequently, it is important to examine the MDZ removal from bioreactor as
348 a function of the initial concentration of antibiotic. Two sets of bioreactors with lower and higher
349 biomass concentrations were spiked with different initial concentrations (1-50 µM) of MDZ as
350 shown in Figure 5. In general, the efficiency of bioreactors decreased with an increase in the
351 initial concentration of MDZ for a fixed period of time. The adsorption phenomena in live
352 biological adsorbent are complex processes, especially when the adsorbate had to stimulate the
353 production of adsorbent. As depicted in Fig.5, only 50.80% of MDZ was removed in a low
354 biomass reactor initially containing 1 µM of MDZ. However, MDZ was completely removed in
355 both low as well as high biomass reactors originally containing 5 µM MDZ in 20 and 18 days of
356 experiments, respectively. Nevertheless, concentrations higher than 5 µM of MDZ showed the
357 same trend as the higher the initial concentration of antibiotic, the lower the removal (%) under
358 constant conditions (same inoculum size and incubation period).

359 It is evident from Table 3 that the EPS production increases with increasing concentration of
360 MDZ in culture; however, this becomes almost constant above 5 µM MDZ. Probably, this was
361 the reason why 1 µM MDZ in low inoculum size test culture was not able to completely remove
362 MDZ, whereas a 5 µM MDZ in low as well as in high inoculum size test cultures were capable
363 to completely remove MDZ from culture media.

364

365 *3.6. Mechanism of MDZ removal*

366 The removal of MDZ from the bioreactor was confirmed by analyzing the liquid phase using
367 HPLC, where a decrease in concentration was recorded. The absence of new chromatographic
368 peaks during the course of the experiments and the respective analysis validated the hypothesis
369 that *C. vulgaris* might not participate in any biodegradation mechanism. To confirm the
370 mechanism, ethyl acetate was used to desorb the adsorbed MDZ from the flocculated biomass.
371 The sole presence of an MDZ peak from the desorbed sample confirmed that MDZ was only
372 removed by sorption mechanism. These results showed that 0.93 mg of MDZ was adsorbed in 1
373 g dry weight of *C. vulgaris*. However, comparing this result with those obtained for MDZ
374 removal per 1 g of dry weight of EPS for the same test bioreactor on the same day (i.e. 0.69 mg
375 MDZ/100 mg of dry weight of EPS in 10 µM MDZ high biomass bioreactor after 20 days of
376 incubation; as mentioned in section 3.4) the amount recovered from dry weight of *C. vulgaris*
377 showed a 9.13% loss in mass balance of MDZ. This decrease in the amount of MDZ suggested
378 its diffusion into the microalgal cell.
379 It is a general observation that dead microalgae cells have a higher adsorption capacity than live
380 cells [54]. Peng et al., [55] demonstrated that live cells of freshwater algae: *S. obliquus* and *C.*
381 *pyrenoidosa* adsorbed less progesterone and norgestrel in aqueous media than their dead
382 biomass. Yu et al., [56] explained that the better adsorption capacity of dead biomass was due to
383 loss of permeability control when the cell membrane structures were destroyed.
384 In this study, the adsorption capacity of live cells with EPS has been compared with live cells
385 without EPS. The first 10 days of culture (Figure 2 (a) and (b)) were considered as cells without
386 EPS while after day 10 cells started to form EPS (especially, attached or bonded) which were
387 considered as live cells with EPS. It is clear from Figure 2 (a) and (b) that there was no removal
388 of MDZ during the initial 10 days (live cells without EPS). The removal of MDZ started with the

389 EPS formation (i.e. after day 10 in all test bioreactors), which proved that live cells with EPS
390 played a major role in MDZ removal.
391 However, to confirm if suspended, attached, or both types of EPS were participating in removing
392 MDZ from water, the removal of MDZ from water was compared against the EPSs (suspended,
393 attached, and total EPS) obtained from 1 µM MDZ test bioreactors (i.e., selected as a
394 representative case) of low and high inoculum (Figure 6). As depicted in Figure 6, it was
395 observed that the attached EPS showed the best fit (i.e., expressed by the highest R^2 value)
396 compared to the other two EPSs (R^2 of suspended EPS=0.49, and R^2 of total EPS=0.88). This
397 high value of correlation coefficient (R^2 = 0.95) of the attached EPS indicated a dominant
398 contribution of MDZ removal and good consistency between the 3D scaffold of the attached EPS
399 and the adsorbed antibiotic.
400
401 3.7. *EC50 of* C. vulgaris *for MDZ*
402 The most common toxicity assay is to calculate the effective concentration (EC50) of a stressor
403 (i.e., metronidazole) at which 50% of the growth rate of an organism (microalgae) is inhibited.
404 Photosynthesis is an essential process for the survival of microalgae, which is related to growth
405 and several other metabolic activities. Thus, any disruption in the growth of microalgae during
406 stressors exposure is related to the changes in chlorophyll (Fig. 7a-b). Antibiotics damage
407 thylakoid and chloroplast lipids as they disrupt membranes and other organelles in cells; thus,
408 destroying the photosystem II (PSII) and consequently chlorophylls and carotenoids [57]. Low
409 chlorophyll content, as a stress response to pharmaceuticals, has been previously observed in
410 microalgae *Tetraselmis chui*, *Isochrysis galbana*, and *C. pyrenoidosa* in the presence of
411 thiamphenicol, florfenicol, and chloramphenicol [58].

412 Two approaches were followed to study the growth inhibition in *C. vulgaris* by MDZ; chronic
413 and acute toxicity of MDZ. The chronic toxicity assay was conducted to assess its adverse effects
414 on an organism as the result of long term exposure, while the acute toxicity is performed for
415 short term exposure. The chronic toxicity of MDZ on *C. vulgaris* was only observed when the
416 antibiotic is added during its growth phase, while acute toxicity was visible when the antibiotic
417 came in contact with microalgal cells at its stationary state. The results showed that the chronic
418 toxicity EC50 for *C. vulgaris* was 83.64 µM (Figure 8a), while for acute toxicity it was 13.63
419 µM (Figure 8b). The growth inhibition of *C. vulgaris* exposed to increasing concentrations of
420 MDZ (i.e., as measured by the chlorophyll-a yield) is shown in Figure 7a-b for chronic and acute
421 toxicity, respectively.
422 According to Commission of the European Community (1996), pollutants with an EC50>100 mg
423 L^{-1} fall under the category of harmless substances, while those with EC50 from 10-100 mg L^{-1}
424 are included under the harmful category. Also, substances with EC50 between 1-10 mg L^{-1}, and
425 EC50<1 mg L^{-1} are considered as very toxic. Results from this study indicate that MDZ is
426 harmful to *C. vulgaris* (Figures 8a-b).
427 Besides the efficiency of *C. vulgaris* for removing DMZ from wastewater, the biomass generated
428 during this treatment would be a valuable feedstock for producing biofuels. Algal biomass has
429 been commonly used as a soil conditioner, plant fertilizer, and animal feed [30]. However, the
430 harvested biomass of *C. vulgaris* would not be recommended as a fertilizer because of its MDZ
431 content. A scheme for biofuel production using MDZ-mediated water is proposed in Figure 9.
432 Rawat et al., [59] have previously demonstrated the production of biogas using algal biomass
433 through anaerobic digestion. The temperatures of these processes vary from 350-800°C [60].

Remarkably, MDZ completely decomposes at 180°C [61], which is significantly lower than the abovementioned lowest temperature of anaerobic digestion processes.

4. Conclusions

In the current study, *C. vulgaris* was used as a novel treatment to remove metronidazole from aqueous media. These results indicated that MDZ stimulated the production of EPS in *C. vulgaris*, which played a major role in the adsorption of this antibiotic. *C. vulgaris* displayed an excellent removal capacity for the target antibiotic at 5μM concentration within the range of 0.05 to 0.5g L^{-1} inoculum of *C. vulgaris*. These results showed that MDZ was only removed by adsorption mechanism onto the scaffold of the EPS, which slowly diffused into the cells. The removal efficiency decreased with increasing initial concentration of antibiotics. The tolerance of *C. vulgaris* towards MDZ depended upon the stage of the life cycle, and on the exposure time to the antibiotic. However, MDZ is marked as a harmful pollutant to *C. vulgaris*. Nevertheless, the recovery of biofuel from the harvested biomass through anaerobic digestion shows promising for future biofuel applications.

Competing interest

The authors declare no competing interest.

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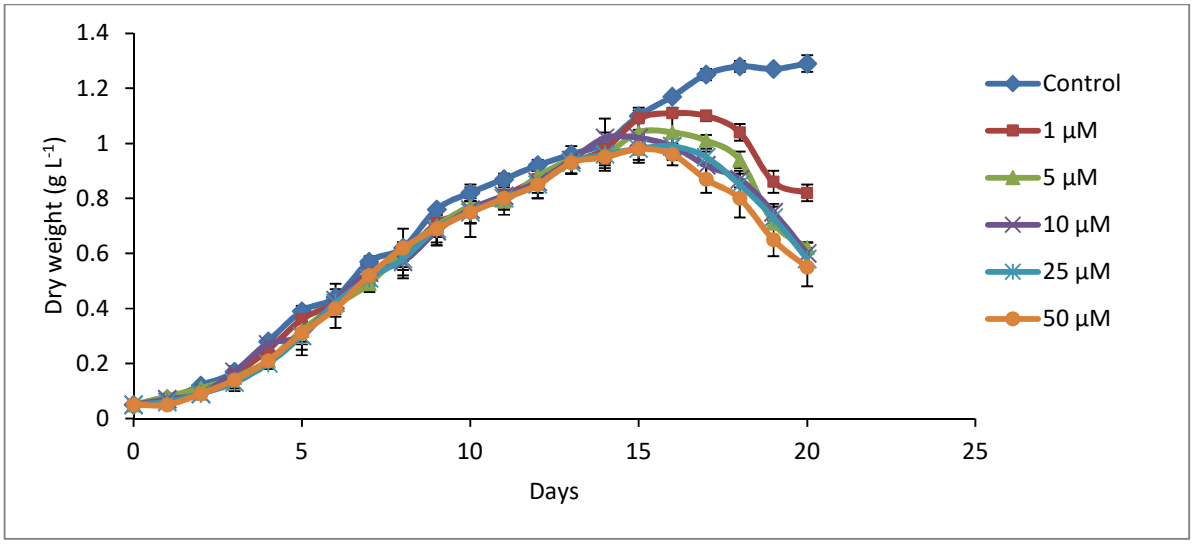


Figure 1a: Growth curve of low inoculum size of *C. vulgaris* for control and test cultures at different concentrations (1-50 µM) of MDZ

- The data are the mean of three replicates.
- Refer Supplementary Table 1a for the data and SD

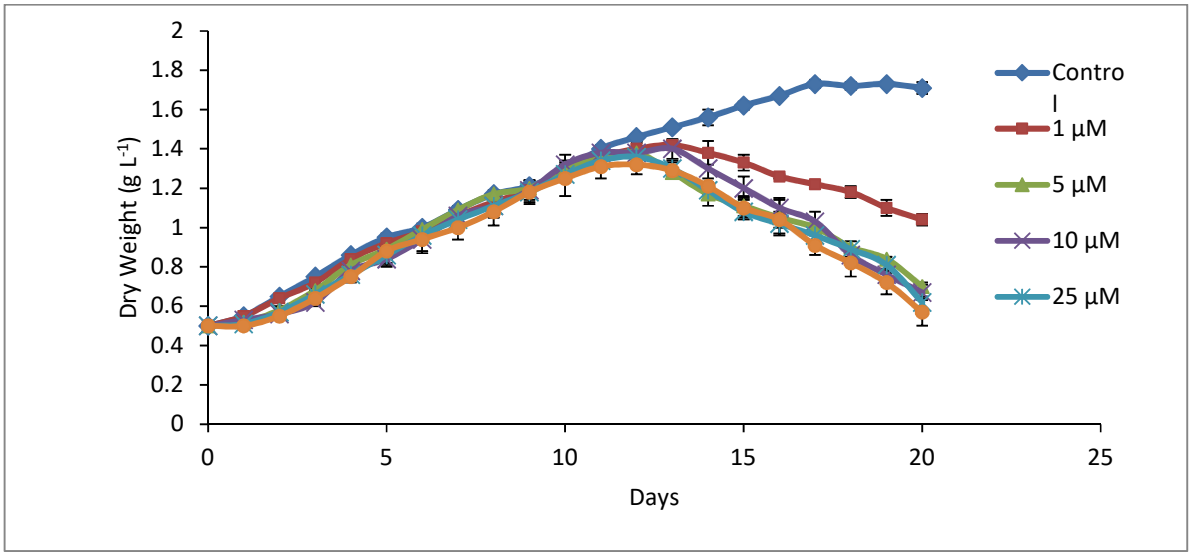


Figure 1b: Growth curve of high inoculum size of *C. vulgaris* for control and test cultures at different concentrations (1-50 µM) of MDZ

- The data are the mean of three replicates.
- Refer Supplementary Table 1b for the data and SD

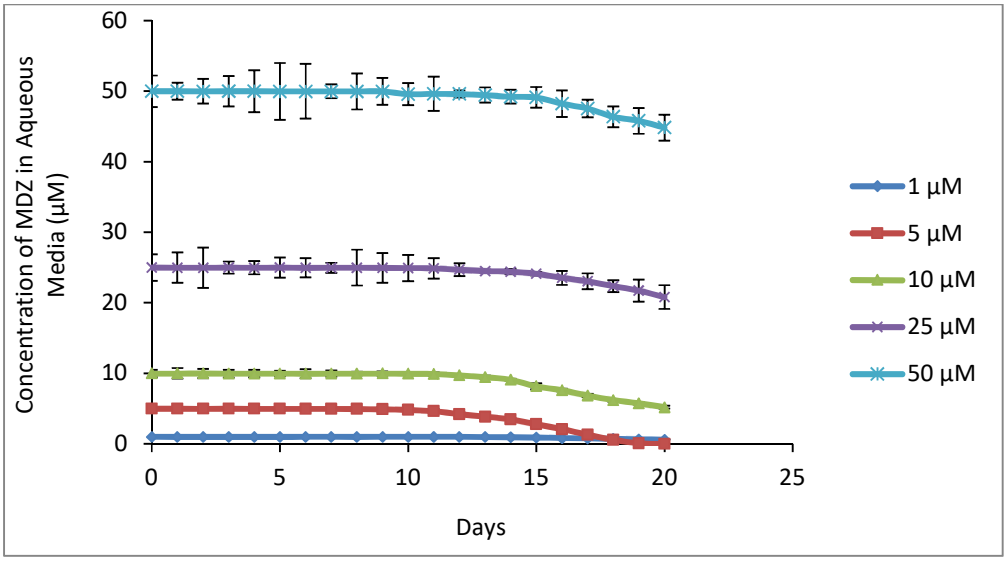


Figure 2a: Evolution of MDZ concentration during treatment using low inoculum size of *C. vulgaris* containing different concentration (1-50 µM) of MDZ in aqueous media

- The data are the mean of three replicates.
- Refer Supplementary Table 2a for the data and SD

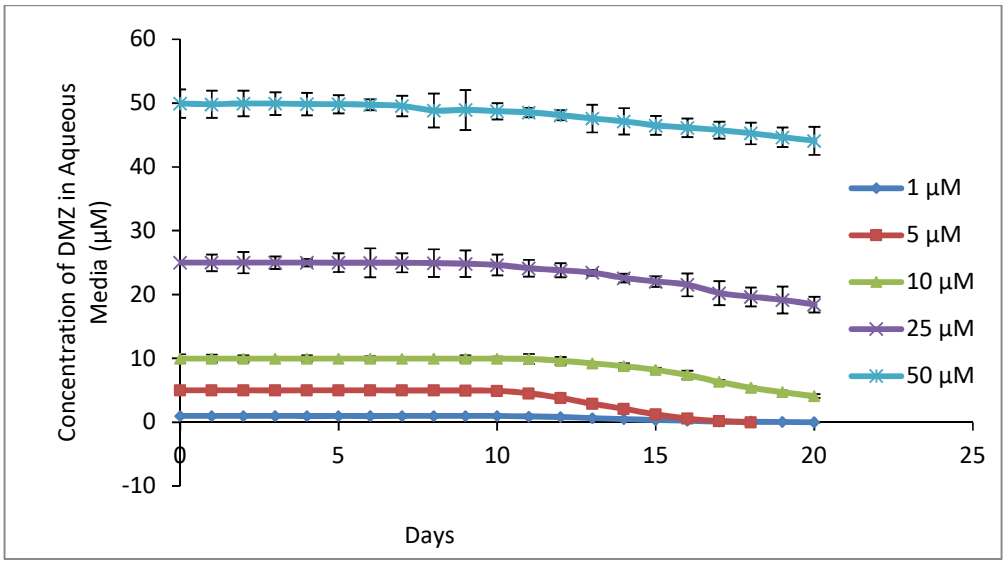


Figure 2b: Evolution of MDZ concentration during treatment using high inoculum size of *C. vulgaris* containing different concentration (1-50 µM) of MDZ in aqueous media

- The data are the mean of three replicates.
- Refer Supplementary Table 2a for the data and SD

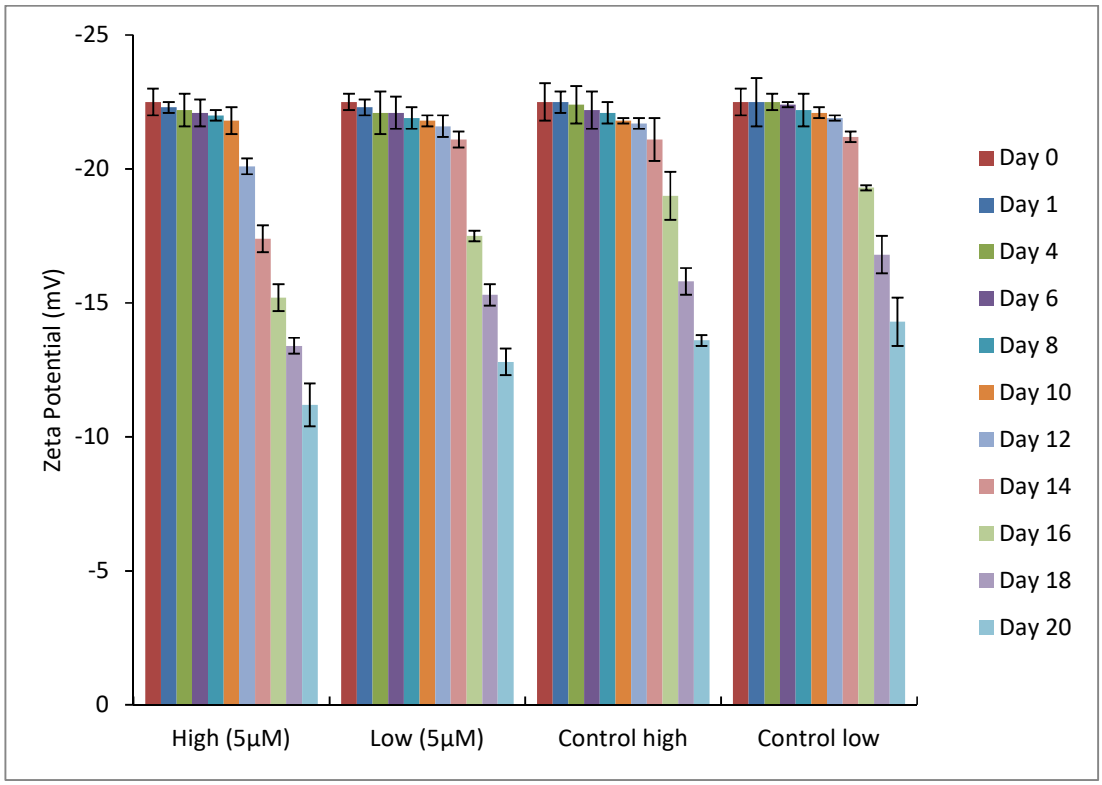
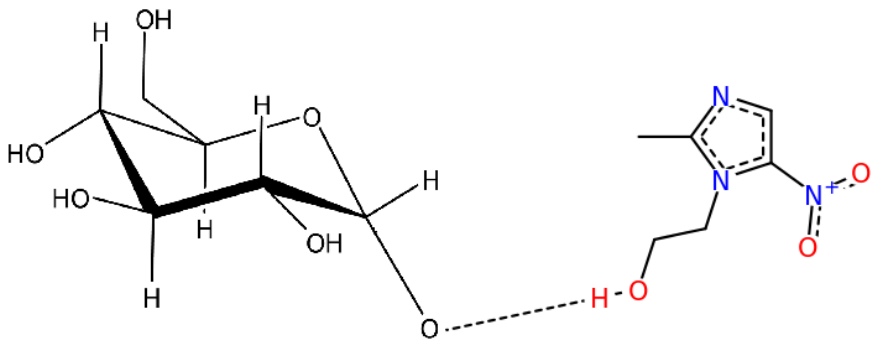
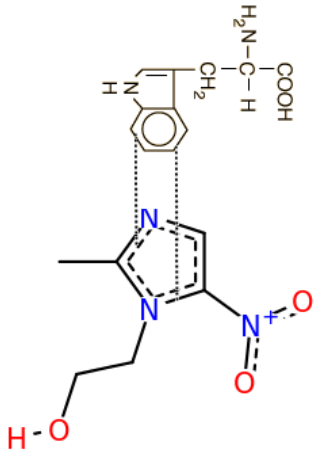


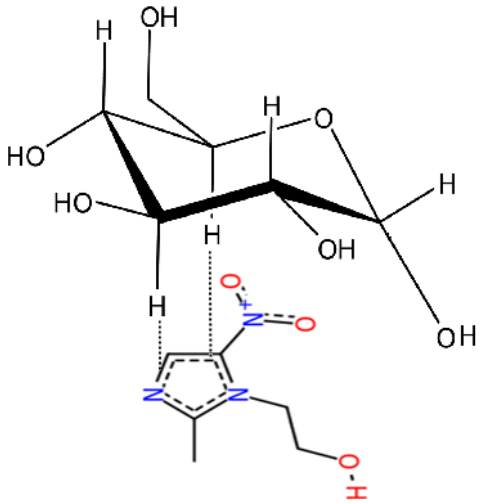
Figure 3: Zeta potential of low and high inoculum size of *C. vulgaris* in control culture and culture spiked with 5 µM MDZ on alternate days



H bond between saccharide and MDZ



π-π dispersion bond between aromatic compound of protein (tryptophan) and MDZ



CH-π bond between saccharide and MDZ

Figure 4: Possible bonding between MDZ and components of EPS

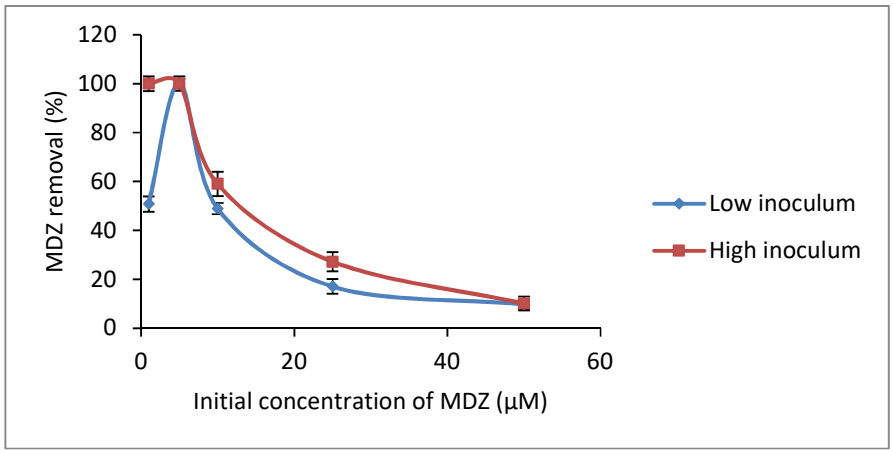


Figure 5: Effect of initial MDZ concentration on MDZ removal

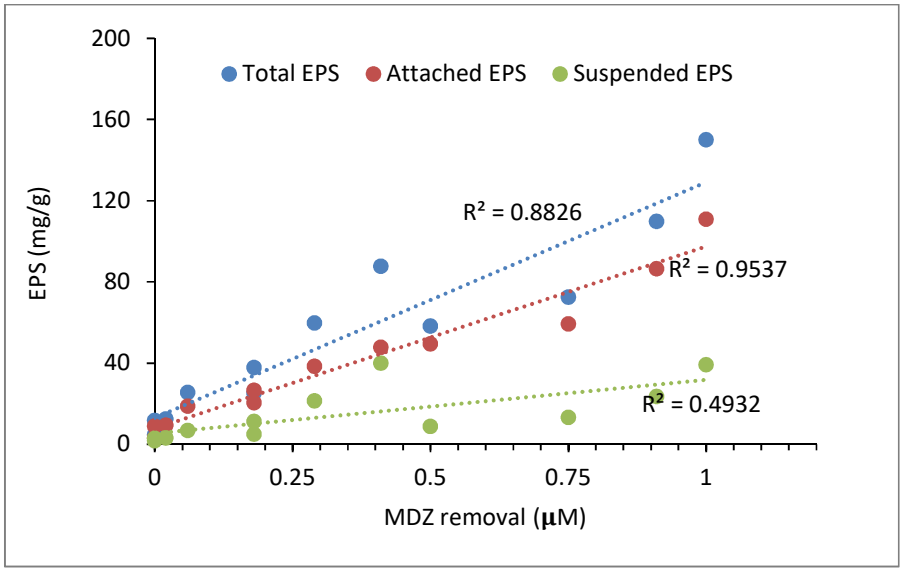


Figure 6: Correlations between the amount of attached, suspended and total EPS with MDZ removal from 1 µM test bioreactors with low and high inoculum from day 10 to day20

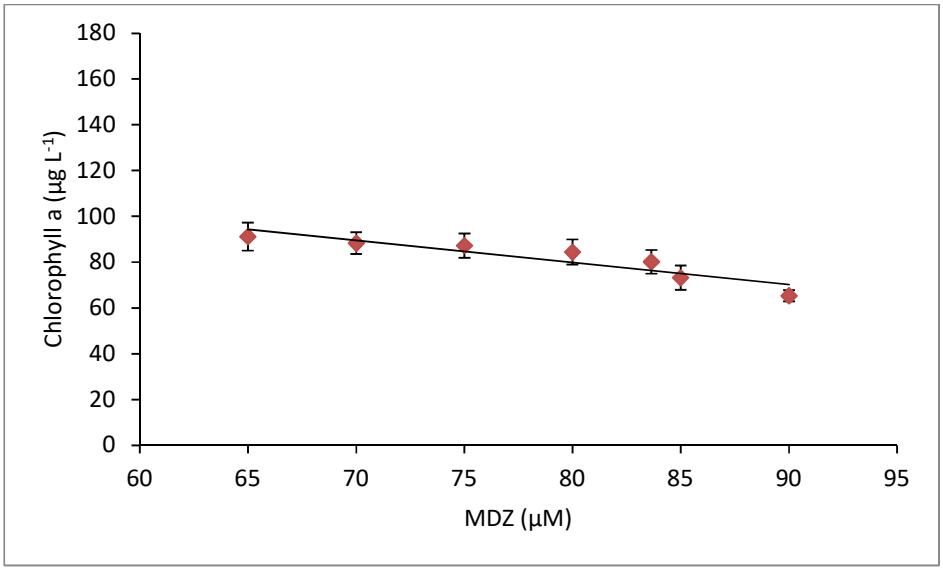


Figure 7a: Chlorophyll a in *C. vulgaris* during a 240-hour metronidazole chronic toxicity test. The control culture (culture without MDZ) possessed 160.22 µg L^{-1} after 240 hours

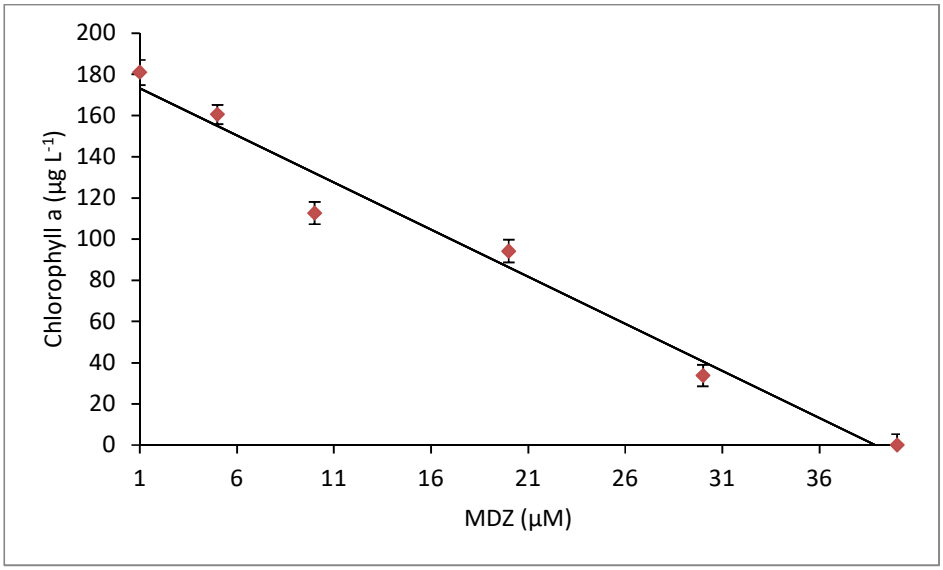


Figure 7b: Chlorophyll a in *C. vulgaris* during a 96-hour metronidazole acute toxicity test. The control culture (culture without MDZ) possessed 240.1 µg L^{-1} after 96 hours

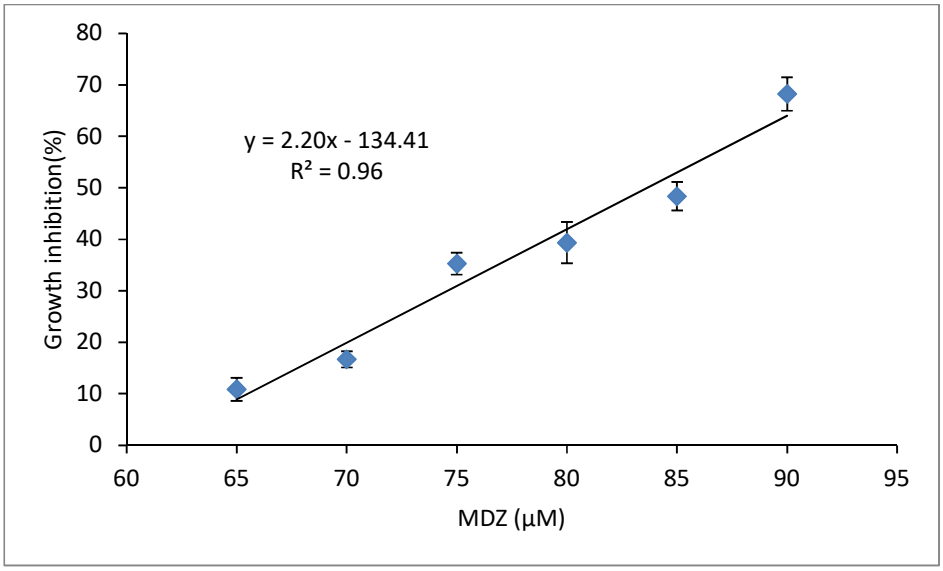


Figure 8a: Growth inhibition (%) of *C. vulgaris* by MDZ during chronic toxicity test.

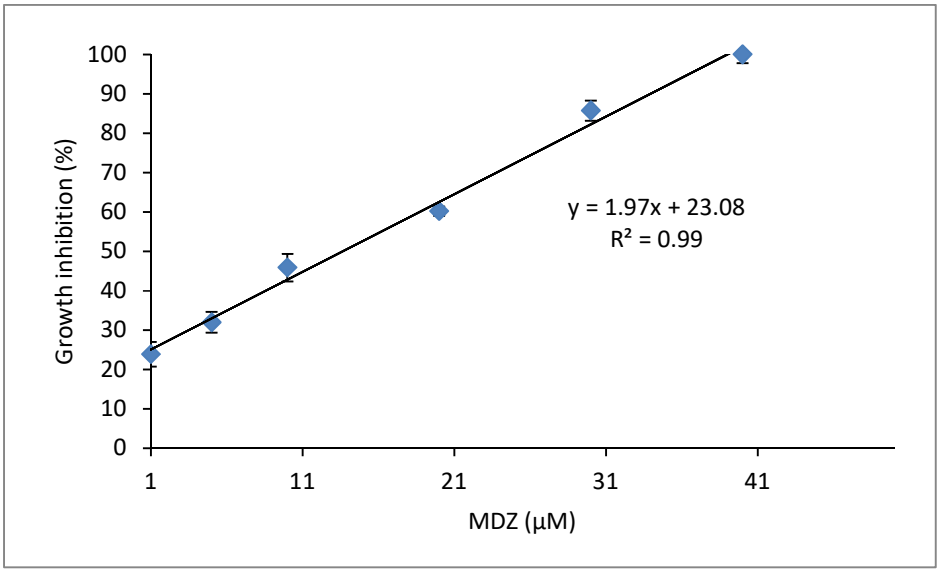


Figure 8b: Growth inhibition (%) of *C. vulgaris* by MDZ during chronic toxicity test

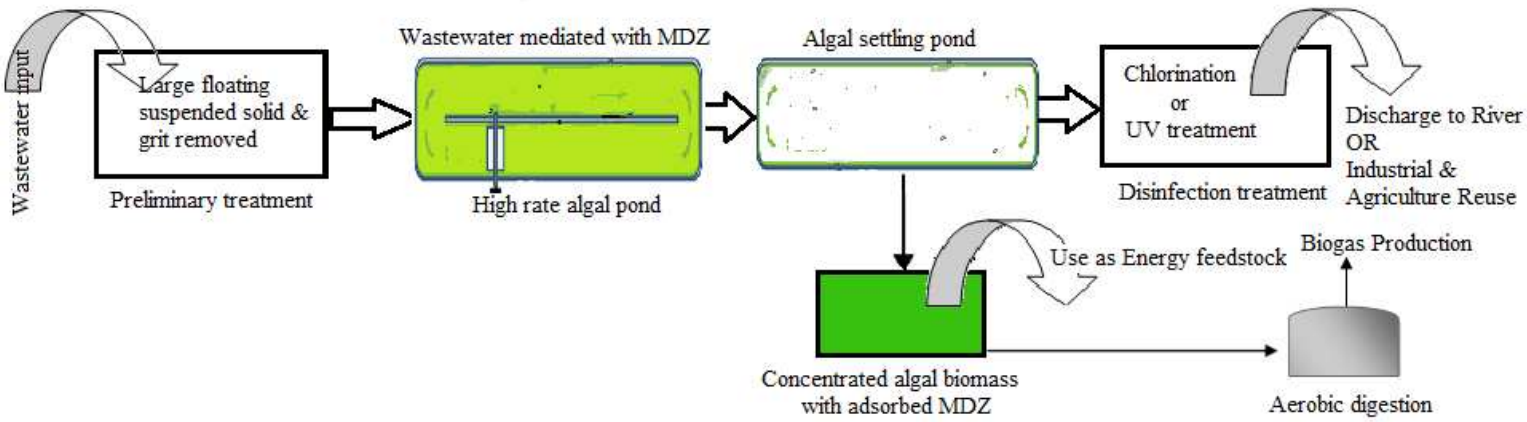


Figure 9: Proposed scheme for biogas production using MDZ-containing wastewater.

Table 1: Compositions of BG-11 media in 1L MilliQ

Chemicals	Weight (mg)
K_2HPO_4	75
KH_2PO_4	175
$MgSO4.7H_2O$	75
$NaNO_3$	250
$CaCl_2.2H_2O$	25
NaCl	25
$Na_2EDTA.2H_2O$	50
$FeSO_47H_2O$ in 0.1mL H_2SO_4	4.9
H_3BO_3	11.42
$ZnSO_4.7H_2O$	1.412
$MnCl_2$. $4H_2O$	0.232
$CuSO_4.5H_2O$	0.252
$Co(NO_3)_2.6H_2O$	0.08
$Na_2MoO_4.2H_2O$	0.192

Table 2: Specific growth rate of *C. vulgaris* in control and test culture with different concentration of MDZ

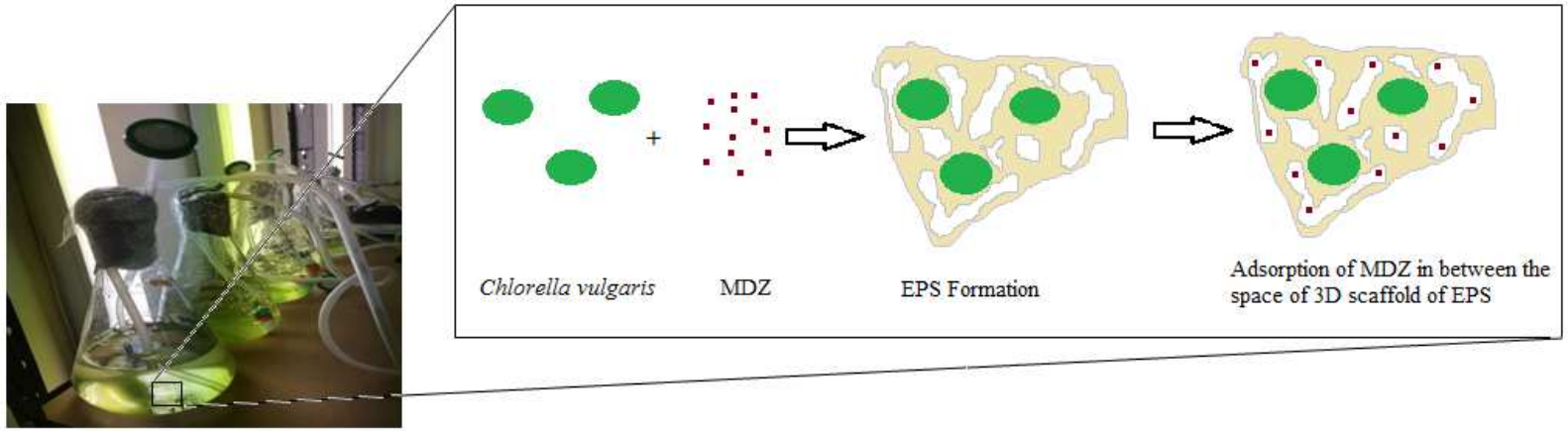
Concentration of MDZ	Low inoculum size		High inoculum size	
	Specific growth rate of early exponential phase (d^{-1})	Specific growth rate of late exponential phase (d^{-1})	Specific growth rate of early exponential phase (d^{-1})	Specific growth rate of late exponential phase (d^{-1})
Control (without MDZ)	0.279	0.055	0.095	0.036
1µM	0.271	0.065	0.094	0.034
5µM	0.273	0.060	0.096	0.029
10 µM	0.272	0.058	0.097	0.019
25 µM	0.271	0.046	0.093	0.035
50 µM	0.271	0.053	0.091	0.028

Table 3: EPS production in control culture and test bioreactors

Days	Control culture		Low inoculum bioreactors									
			1μM		5 μM		10 μM		25 μM		50 μM	
	EPS (S)*	EPS (A)*	EPS (S)	EPS (A)	EPS (S)	EPS (A)	EPS (S)	EPS (A)	EPS (S)	EPS (A)	EPS (S)	EPS (A)
10	2.31±0.11	1.45±0.09	1.62±0.33	3.01±0.08	1.71±0.08	5.54±0.16	1.76±0.63	5.12±0.53	1.97±0.16	5.62±0.11	1.88±0.09	6.32±1.66
12	7.40±0.26	7.92±0.22	2.89±0.78	8.84±0.65	2.19±0.22	15.33±1.11	2.11±0.08	15.54±0.38	2.21±0.36	15.78±0.76	2.08±0.23	16.34±1.89
14	20.52±0.46	14.91±0.28	6.79±0.54	18.77±0.54	4.23±0.23	37.87±1.29	4.19±0.43	37.1±0.97	4.56±0.69	38.2±1.06	4.21±0.25	38.33±1.27
16	34.82±0.25	19.28±0.78	11.19±1.09	26.54±0.43	7.88±0.18	76.45±2.66	7.12±0.65	77.15±1.86	7.19±0.46	75.29±1.64	6.89±0.61	73.32±2.76
18	42.95±1.67	30.65±0.64	21.32±1.68	38.41±0.79	20.65±	113.62±3.54	19.9±1.06	111.3±1.69	14.06±0.87	121.23±1.39	18.88±0.28	110.12±3.18
20	48.76±1.86	37.50±2.09	39.98±1.87	47.72±1.23	33.67±1.26	146.1±2.34	31.04±0.38	142.32±2.21	24.76±1.11	148.99±2.43	30.03±0.53	139.31±5.16
High inoculum bioreactors												
10	4.27±0.22	2.11±0.13	3.12±0.08	9.32±0.65	3.25±0.11	12.25±1.07	3.29±0.38	12.11±1.09	3.41±0.09	12.43±1.66	3.52±0.33	13.11±0.87
12	9.16±0.31	8.99±0.76	4.89±0.18	20.36±0.23	4.22±0.18	26.48±1.76	4.15±0.79	25.54±1.24	4.55±0.97	26.98±1.45	4.23±0.27	27.23±1.56
14	29.12±1.55	21.34±1.56	8.77±0.54	49.42±1.43	6.78±0.16	55.15±1.18	7.12±0.68	57.75±1.76	7.67±0.76	56.67±2.12	7.45±0.87	58.43±0.98
16	37.45±1.42	34.77±1.82	13.23±0.21	59.23±1.54	11.32±0.64	107.92±2.45	12.34±0.45	78.92±2.06	12.65±1.14	76.78±2.32	10.54±1.09	76.67±1.67
18	48.72±2.64	46.34±2.44	23.45±0.67	86.43±2.69	14.43±0.98	121.4±2.27	13.98±0.88	119.12±2.46	21.33±1.12	110.43±2.34	13.78±0.95	118.34±2.65
20	54.66±1.98	51.86±1.87	39.18±2.11	110.9±2.45	26.76±1.17	151.6±3.45	25.08±1.11	148.78±2.65	32.65±1.45	140.01±4.11	24.44±1.22	145.55±3.56

(S)*—Suspended EPS; (A)*--Attached EPS; EPS were measured in mg g^{-1} dry weight of *C. vulgaris*

Graphical Abstract:



Schematic diagram of MDZ removal in Chlorella vulgaris culture