Biological activated carbon process for biotransformation of azo dye Carmoisine by *Klebsiella* spp.

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**ABSTRACT**

The feasibility of employing the biological activated carbon (BAC) process to debilitate azo dye Carmoisine by *Klebsiella* spp. was investigated. Plate assay revealed the capability of *Klebsiella* spp. for removal of Carmoisine via degradation. Kinetic parameters were measured for Carmoisine debilitation by *Klebsiella* spp. using the suspended anaerobic process. Two types of granular and rod-shaped activated carbon were used to form the biological beds in order to study the Carmoisine debilitation in batch processes. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were used to indicate the colonization and biofilm formation of bacteria grown on activated carbon particles (ACPs). Thin-layer chromatography (TLC), liquid chromatography–mass spectrometry (LC–MS), high-pressure liquid chromatography (HPLC) and biosorption studies demonstrated biotransformation of Carmoisine into its constituent aromatic amines during the Carmoisine debilitation in suspended anaerobic and BAC processes. The porosity of activated carbons, inoculation size and age of biological beds were the important factors affecting the viability of bacterial cells grown on ACPs and, consequently, the rate and efficiency of the Carmoisine debilitation process determined through spectrophotometry. The reusability of biological beds was demonstrated by conducting sequential batch experiments. In conclusion, the BAC process proved to be an efficient method for anaerobic dye degradation.

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Introduction

Azo dyes with one or more –N=N– group(s) are highly stable synthetic dyes and, therefore, have long been utilized by many industries. However, structural stability renders azo dyes a threat to the environment, which requires these dyes to be eliminated during wastewater treatment before discharge into water bodies or wetlands [1]. In order to ensure the efficient removal of dyes from wastewater effluents, wastewater treatment plants have been encouraged to utilize recommended biological treatments. Due to the various advantages of these treatments such as being environmentally friendly, not requiring significant investment in equipment and supplies, lower sludge production as well as being safe, flexible and easily handled [2,3], biological methods have proved to be a promising alternative to traditional methods in wastewater dye treatments [4].

Previous studies have shown that anaerobic treatment as an effective dye biodegradation method [5,6] was very slow [2,7], resulting in an increase in solid retention time, working volume and construction investment for the bioreactor [8]. Therefore, the development of strategies to enhance the rate of efficient biological dye treatments is necessary. In this regard, the use of potent microorganisms in the biodegradation process and proper process design for the use of a maximum capacity of microorganisms can be an effective strategy to compensate for the low rates of anaerobic wastewater treatments.

Attached growth systems, compared with suspended growth systems, render an increased treatment capacity in a compact form with an area requirement which is a fraction of that of their suspended counterparts. Attached growth processes include microorganisms growing in attachment to inert packing materials utilized in well-known reactors of fluidized bed (FB) and anaerobic filter (ANF) for anaerobic conversion of organic matters [9]. In a number of studies, the term ‘attached-growth process’ has been replaced by ‘immobilized-cell system (ICS)’ [10,11]. Although this term does not seem reasonable when proposed against a suspended system, it was frequently used in earlier studies. Immobilized cell systems or fixed-film bioreactors exhibiting improved efficiencies in decolourization processes due to dense biomass, increased stability against shock loadings contributing to higher rates of hydraulic loading, and the production of less biomass sludge, supported the survival of slow-growing microorganisms and provided more optimal conditions for both aerobic and anaerobic activities [10–12], rendering a reusable immobilized matrix, resulting in a more cost-efficient procedure [13] and providing greater available surface area for the adsorption of dye [14].

Commonly studied ICS in decolourization processes dealt with microorganisms available in the adsorbed form in porous media or foam, or entrapped in different gel beads [11,15]. Alginate beads, polyethylene and polyurethane foams, silica matrices and nanoparticles such as iron-oxide are the most commonly studied materials for immobilization of microorganisms used in azo dye decolourization [13,14,16–23]. In this regard, activated carbon due to surface roughness and the high specific area provides superior features for the rapid colonization of microorganisms and biofilm expansion by means of surface niches providing shelter against the shear forces [12]. Therefore, since the 1970s, two processes, namely, PACT (Powdered Activated Carbon Treatment) and BAC (Biological Activated Carbon), integrating high adsorption of activated carbon with biodegradation, have received increased attention in wastewater treatment studies [24,25]. The synergistic factors of increased biodegradation and adsorption make the BAC process promising for anaerobic biodecolourization processes [26]. However, only a few studies on the use of BAC-process have been reported in the biological removal of azo dyes [7,12,25,27,28]. This study, therefore, aims to evaluate the biological activated carbon process for the biodegradation of azo dye Carmoisine using Klebsiella spp. in batch experiments. Two types of activated carbon, granular and rod-shaped, were used to support the attached growth of bacteria and formation of biofilm bed. Bio-transformation and kinetic studies were performed to determine the mechanism and yield of the process.

It is worth noting that, although ‘decolourization’ is a common term in the literature focused on the elimination of dyes from a medium, it is etymologically incorrect, since the medium does not turn completely white or colorless. Moreover, ‘dye removal’ is likewise not an accurate characterization when the process undergoes dye degradation. Therefore, the term ‘dye debilitation’ has been suggested here as a more accurate description of what the dye molecules undergo during degradation or transformation in the treatment process.

2. Materials and methods

2.1. Chemicals

All chemicals and culture medium components were of analytical grade and prepared from Merck KGaA (Germany), except items otherwise indicated. Sucrose was of commercial grade; white sugar (Pardis brand) was acquired from Golestan Co. (http://www.golestan.
2.2. Activated carbon properties

Commercially activated carbon in two forms of granular (Jacobi, AquaSorb 2000, 8 x 30 mesh, Sweden) and rod-shaped or extruded (Jacoba, XH 4 mm, China) with iodine numbers of 950 and 900 mg/g, respectively, were purchased.

The surface area was estimated for both granular and rod-shaped activated carbons (GAC and RAC) using nitrogen gas adsorption analysis at 77 K with a BELSORP-mini II. Prior to gas adsorption measurements, GAC and RAC samples were degassed with nitrogen gas at 150°C for 4 and 6 h, respectively. Analysis was conducted using the BEL Master™ programme. The surface morphology of the activated carbon samples was studied using a scanning electron microscope (Vega3, Tescan) with an acceleration voltage of 20.0 kV. Elemental composition was analysed for each sample using the Energy dispersive X-ray spectroscopy (EDS) technique. The surface functional groups were determined by Fourier Transform infrared (FTIR) spectroscopy. The FTIR spectra were recorded between 400 and 4500 cm⁻¹ using a spectrometer (Nicolet Nexus 870, USA).

2.3. Microorganism and cultivation

Klebsiella spp. isolated from expired date palm liquid with a GenBank accession number of KY357316 was used in this study. Bacteria were grown on a solid medium of YTS including yeast extract, 1% (w/v); tryptone (casein hydrolysate), 2% (w/v); sucrose, 2% (w/v) and agar, 2% (w/v) at 30°C for 23–24 h. Bacterial suspension used throughout the study was prepared by the cultivation of Klebsiella spp. in YTS broth medium incubated for 19–21 h at 30°C and under shaking conditions (180 rpm). The suspension was then assessed for a number of viable cells as colony-forming units (CFUs) per millilitre of culture.

2.4. Plate assay

Klebsiella spp. was initially evaluated for the debilitation potential of Carmoisine through an agar plate test. To this end, 100 µl of bacterial suspension (≈250 × 10⁶ CFU/ml) was streaked on a solid medium of YTS°C including YTS composition and Carmoisine at a concentration of 50 mg/l. Plates were incubated at 30°C and monitored daily for visual changes in colour in the culture medium. When no dye was visually observed in plates, microbial biomass on the plates’ surface was collected and suspended in normal sterile saline to be analysed for biosorption and/or bioaccumulation of Carmoisine according to procedures described in the Analytical Methods section.

2.5. Assessment of Carmoisine debilitation in suspended anaerobic process

Debilitation of azo dye Carmoisine by Klebsiella spp. was evaluated in an anaerobic process. To this end, 90 ml of YS°C,50ppm medium in a 100-ml Duran bottle was inoculated with cells of 4.5 ml Klebsiella spp. preculture (19–21 h grown, ≃250 × 10⁶ CFU/ml) and incubated at 30°C without shaking. At zero time of incubation as well as at specified time intervals, samples were withdrawn and centrifuged (16,060g, for 10 min) to eliminate the bacterial pellets. The supernatants were subsequently subjected to spectrophotometric analysis, thin layer chromatography and sucrose measurements according to procedures described in the ‘Analytical methods’ section. In addition, the biotransformation of Carmoisine during the anaerobic process was investigated by HPLC analysis via methods also described in the ‘Analytical methods’ section. Moreover, cell pellets collected at the end of the process were resuspended in sterile normal saline to be examined for biosorption and/or bioaccumulation of Carmoisine into bacterial cells.

Furthermore, various amounts of cells harvested from 4.5, 50 or 100 ml of preculture were used for the inoculation of suspended anaerobic processes. Samples were taken and analysed using spectrophotometry and thin layer chromatography. In addition, a Carmoisine-debilitated sample from the anaerobic process with cells of 100 ml preculture was applied bandwise on a TLC plate. The fluorescent bands were scraped off and analysed using liquid chromatography–mass spectrometry (LC–MS).

2.6. Biological activated carbon beds in the debilitation of Carmoisine

Anaerobic debilitation of Carmoisine was investigated in a system of carbon-attached bacteria. Activated carbons (ACs), granular or rod-shaped, were used to establish a bed of biologically activated carbon. In order to accomplish this, 75 ml YTS was added to a 100-ml Duran bottle containing 17 g of activated carbon. Then, each bottle was inoculated with Klebsiella spp. cells harvested from specified sizes (4.5, 50 ml or 100 ml) of cell suspension (≈250 × 10⁶ CFU/ml) and statically incubated at 30°C for 10 days when the biomass was visually observed.
on the surface of ACs. On days 0, 3, 5, 7 and 10, several particles of activated carbon were taken using a pre-sterilized spatula and assessed for viable cells as CFU per gram of ACs according to the method described below.

Moreover, the bacteria colonisation and biofilm formation on beds inoculated with cells of 4.5 ml preculture were investigated. To this end, AC samples from 3-, 5-, 7- and 10-day bed were glutaraldehyde-fixed and studied by scanning electron microscopy (SEM). In addition, 3-day samples were further investigated by confocal laser scanning microscopy (CLSM). Procedures are explained in further detail in the ‘Analytical Methods’ section.

Five-day and 10-day beds were compared for Carmoisine removal in an anaerobic process. To accomplish this, the liquid phase on bed was gently discharged and replaced by 75 ml YS+C,50ppm and then incubated statically at 30°C. Samples were prepared and analysed through spectrophotometry and TLC to evaluate the Carmoisine debilitation. Moreover, the effective rate ($t_{\text{eff},d}$) and effective efficiency ($E_{\text{eff},d}$) of debilitation were estimated using equations Equations (1) and (2) for all conditions (Table 1).

$$t_{\text{eff},d} = \frac{C_{\text{medium}} - C_{\text{eff}}}{t_{\text{eff}}}$$  \hspace{1cm} (1)

$$E_{\text{eff},d} = \frac{C_{\text{medium}} - C_{\text{eff}}}{C_{\text{medium}}} \times 100$$  \hspace{1cm} (2)

where $C_{\text{medium}}$ (mg l$^{-1}$) and $C_{\text{eff}}$ (mg l$^{-1}$) are the Carmoisine concentrations in YS$^{+}$C,50ppm medium before inoculation and at the effective time of process, respectively, and $t_{\text{eff}}$ is the effective time of process indicating the effective time at which TLC analyses showed the disappearance of the Carmoisine spot (h).

Moreover, the biotransformation of Carmoisine during the anaerobic process using the five-day AC-beds was investigated by HPLC analysis, as described in the ‘Analytical Methods’ section. Control experiments were also performed using non-inoculated AC-beds, and Carmoisine debilitated samples were assessed by HPLC analysis.

### 2.7. Biotransformation studies

High-pressure liquid chromatography (HPLC) was used to investigate the biotransformation of Carmoisine into its aromatic amine constituents. For this purpose, YS medium, YS including 4-aminonaphthalene-1-sulfonic acid at concentrations 50 mg/l (YS$^{+}$A,50ppm) and 100 mg/l (YS$^{+}$A,100ppm) and YS including Carmoisine at concentrations 50 mg/l (YS$^{+}$C,50ppm) and 100 mg/l (YS$^{+}$C,100ppm) were prepared as the standards in HPLC studies. Samples from the standard media and Carmoisine-debilitated YS$^{+}$C,50ppm from the suspended and attached-growth processes were prepared. Solvent-extracted samples were analysed through HPLC analysis, as described in the ‘Analytical Methods’ section.

### 2.8. Sustainability of the biological bed of activated carbon in repeated processes

Bacterial bed of activated carbon was used in seven consecutive batches of the debilitation process to evaluate the long-term activity and reusability of AC-adhered Klebsiella spp. in the anaerobic process. At the beginning of each batch of Carmoisine debilitation, depleted medium was replaced by fresh YS$^{+}$C,50ppm. Before the 6th and 7th batches, beds were incubated in YTS for 48 h to recover the viability of the beds. During the experiments, cell-free samples were analysed with spectrophotometry and TLC to measure the Carmoisine debilitation in each batch. In addition, viable cells on carbon bed were estimated as CFU per gram of ACs at the end of each batch.

### 2.9. Analytical methods

#### 2.9.1. Spectrophotometry approach

Carmoisine concentration was estimated through the spectrophotometry method. The absorption spectra of samples were recorded via a T80+UV/Vis spectrometer (PG Instruments Ltd.) in the range of 200–800 nm. The absorbance at the maximum absorption wavelength of Carmoisine ($\lambda_{\text{max}} = 515$ nm) was used to determine the concentration of Carmoisine using a calibration curve [29]. Percent of Carmoisine debilitation (CD%) was

| Table 1. Total rate ($r_{\text{tot},d}$) and total efficiency ($E_{\text{tot},d}$) of Carmoisine debilitation using 5-day and 10-day granular (G) and rod-shaped (R) beds inoculated with Klebsiella spp. cells of 4.5, 50 and 100 ml preculture. |
|-----------------------------------------------|
| S-Day beds | G4.5 | G50 | G100 | R4.5 | R50 | R100 |
|----------------|------|-----|------|------|-----|------|
| $t_{\text{eff}}$ (h) | 6 | 8 | 6 | 8 | 6 | 8 |
| $r_{\text{eff},d}$ (mg l$^{-1}$ h$^{-1}$) | 8.27 ± 0.16 | 6.28 ± 0.12 | 6.21 ± 0.12 | 8.01 ± 0.16 | 7.44 ± 0.14 | 5.93 ± 0.11 |
| $E_{\text{eff},d}$ (%) | 98.72 ± 1.28 | 99.98 ± 0.01 | 98.87 ± 1.12 | 95.69 ± 1.91 | 88.84 ± 1.77 | 94.47 ± 1.88 |
| 10-Day beds | | | | | | |
| $t_{\text{eff}}$ (h) | 6 | 6 | 6 | 4 | 4 | 4 |
| $r_{\text{eff},d}$ (mg l$^{-1}$ h$^{-1}$) | 8.43 ± 0.16 | 8.41 ± 0.16 | 8.44 ± 0.17 | 9.52 ± 0.19 | 9.81 ± 0.19 | 10.53 ± 0.21 |
| $E_{\text{eff},d}$ (%) | 98.14 ± 1.76 | 98.01 ± 1.85 | 98.34 ± 1.59 | 73.94 ± 1.47 | 76.14 ± 1.32 | 81.77 ± 1.63 |

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determined as \((\text{C}_{\text{medium}} - C_t)/\text{C}_{\text{medium}}\) × 100, where \(\text{C}_{\text{medium}}\) (mg l\(^{-1}\)) and \(C_t\) (mg l\(^{-1}\)) are Carmoisine concentration in YS+5C,50ppm medium before inoculation and after \(t\) (h) of incubation.

2.9.2. TLC, HPLC and LC-MS analyses

Aqueous samples were extracted three times with an equal volume of n-butanol. Extracts were pooled and concentrated in an Eppendorf Concentrator 5301 to purge the solvent. The concentrated residue was then subjected to further analysis. The residue was examined by thin layer chromatography on fluorescent silica gel plates using a mobile phase of butanol:water:acetic acid (2:2:1) [29], and resolved plates were observed under a UV illuminator (Vilber Lourmat, CN-15MC, France).

HPLC analysis was performed on ACME 9000 (Young-lin Instruments Inc., Korea) equipped with two columns of Hector-M C18 (25 cm × 4.6 mm, 5 μm and 15 cm × 4.6 mm, 5 μm, Rstech, Korea) in series. The mobile phase was a combination of solvent A (0.2% phosphoric acid in water) and solvent B (methanol:acetonitrile, 50:50) in a defined gradient programme starting with 98% solvent A for 14 min. The elution programme was followed by 82% solvent A for 13 min and then 98% solvent A for 5 min. The HPLC flow rate was 0.7 ml/min. The eluates were monitored by UV absorption (UV/VIS Detector, Acme 9000) at 250 nm.

The mass analysis was accomplished using an Agilent 6410 Triple Quadrupole MS equipped with an electrospray ionisation (ESI) source. The samples were injected into the reverse phase C18 (250 × 4.6 mm, 5 μm) HPLC column using an autosampler. Elution was carried out using HPLC grade methanol as a mobile phase. The column was run at a flow rate of 1 ml/min for 10 min at room temperature. The eluates were monitored at a wavelength of 254 nm using a UV–vis detector and conducted into the mass spectrometer (MS) via capillary. The MS operating conditions were as follows: negative ion mode, scan spectra from m/z 100–1700 Da, drying gas (N\(_2\)) with a flow rate of 6.0 l/min, a drying gas temperature of 325°C, a nebuliser pressure of 25 psi, a capillary voltage of 3500 V, a skimmer voltage of 65 V and a fragmentor voltage of 65 V. The sample collision energy was set at 0 V. Instrumentation control of data acquisitions were performed with MassHunter Workstation Data Acquisition Software Ver. B.01. 03 [29,30].

2.9.3. Sucrose measurements

Sucrose consumption was estimated during the Carmoisine debilitation processes. In order to break the sucrose into reducing sugars, samples were diluted and hydrolysed according to a slightly modified approach of Miloski et al. [31] Briefly, 50 μl of concentrated HCl was added to 500-μl samples and heated in a boiling water bath for 15 min. The samples were subsequently cooled under a running tap water to an ambient temperature, and after neutralisation with NaOH (6 N, 90 μl), the volumes were adjusted to 1 ml with distilled water. Then, reducing sugars were measured using the dinitrosalicylic acid (DNS) method developed by Miller [32], and the results were converted into sucrose concentration (as percent).

2.9.4. Enumeration of viable bacteria in activated carbon granules

A count of attached bacteria on AC samples was estimated through the plate count method following the detachment treatment of bacteria. To do this, AC samples were washed with saline, weighted and homogenised in a blending solution (Tween 80, 0.1%; EDTA, 0.05%; Tryptone, 0.1%; 0.01 M Tris buffer, pH = 7) at 14,000 rpm (Omni-Mixer model 17,150, Dupont/ Sorvall) for 3 min at 4°C according to a slightly modified version of the method described by Camper et al. [33] The viability of desorbed bacteria in buffer solution was estimated as colony-forming units (CFUs) through the plating of serial dilutions on YTS-agar medium and reported as CFU per gram of ACs.

2.9.5. Microscopic observations of bacteria in activated carbon granules

The carbon particles with attached bacteria were fixed by glutaraldehyde and dehydrated in a set of ethanol solutions according to a method adapted from Vatandoostarani et al. [3] for microscopic investigations. Fixed samples were microscopically examined for bacteria attached to the AC particles using scanning electron microscopy (SEM) using VEGA3\™TESCAN SEM, and micrographs were provided. The fixed AC samples were stained with RealQ Plus 2× Master Mix Green with High ROX™ (Denmark). After staining, fluorescent images were provided using a confocal laser scanning microscope (CLSM, Leica TCS SP2, Heidelberg, Germany) equipped with two objectives (10×/0.4 and 63×/1.2) and detectors and filter sets for monitoring green-stained bacteria. RealQ Plus 2× Master Mix Green contains a fluorescent dye that permeates into the bacterial cell to bind with DNA which is stained green. For this purpose, fixed samples were incubated with 100 μl of stain solution (1×) at room temperature for 30 min. Excitation was provided by an Ar laser with line at 364 nm, using 399–553 nm for emission. CLSM images were generated using the assistant software Huygens Essential 3.7.1p2 64b (Scientific Volume Imaging B.V.).
2.9.6. Biosorption/bioaccumulation test

*Klebsiella* spp. biomass was assessed for any adsorbed or accumulated Carmoisine in cells during the Carmoisine debilitation process. In order to accomplish this, biomass was treated with cold methanol to extract the internal metabolites according to a slightly modified method described by Foschi et al. [34]. In brief, 1 ml of bacterial suspension in normal saline was centrifuged at 10,000 rpm (16,060g, for 10 min) using a microcentrifuge (Heraeus Biofuge Fresco, Germany). Bacterial pellets were resuspended in a 500 μl mixture of 100% cold methanol:sterile saline (1:1) (4°C), vigorously mixed (vortex, 1 min), and subsequently frozen at −80°C for 30 min and then thawed on ice for 30 min. The freezing–thawing steps were repeated three times in order to leak the metabolites from the cells. The suspension was then centrifuged at 10,000 rpm (16,060g, for 10 min) and the supernatant was transferred to a separate microtube. The pellets were again subjected to the above-mentioned procedure, and then, the two supernatants were combined. The pooled supernatant was scanned by UV–vis spectrophotometry and placed into an Eppendorf Concentrator 5301 to purge the methanol. Then, the aqueous residue was extracted using n-butanol and analysed via thin layer chromatography. For comparison, methanol:saline (1:1) including 50 mg/l Carmoisine (Met-Sal+C,50ppm) was used in both spectrophotometry and TLC analyses.

2.10. Statistical analysis

All experiments were conducted in triplicate. Data are expressed as the mean ± standard deviation of three independent experiments, each performed in triplicate. The graphs represent error bars with plus and minus one standard deviation from the mean.

3. Results and discussion

3.1. Activated carbon characteristics

Nitrogen adsorption/desorption isotherms were used to estimate the Brunauer, Emmett and Teller (BET) surface area and Barrett–Joyner–Halenda (BJH) pore size of specimens. BET surface area and BJH pore volume and pore area were determined as the triplicate set of ‘900.37 m²/g, 0.1456 cm³/g; 156.14 m²/g’ and ‘940.07 m²/g, 0.099584 cm³/g, 90.958 m²/g’ for the GAC and RAC samples, respectively. Energy dispersive X-ray spectroscopy (EDS) showed the profile of atomic percentages as ‘C (90.09), O (6.00), Al (0.86), Si (0.80) and S (2.25)’ and ‘C (92.41), O (7.17), Si (0.32) and Ca (0.1)’ within the GAC and RAC samples, respectively. Carbon was the dominant element, and oxygen was ranked second. Other impurities were attributed to the origin from which the activated carbon was produced [35,36]. The surface functional groups on the activated carbon samples were identified by FTIR spectra (Supplementary Figure S1) and elucidated using IRPal software (Version 2) and descriptions by Demiral et al. [37]. A broad absorption band in the region of 3500–3400 cm⁻¹ may be attributed to the O–H stretching vibrations of hydroxyl functional groups. The bands around 2920–2850 cm⁻¹ are attributed to C–H asymmetric stretching. Absorption at 2370–2340 cm⁻¹ is ascribed to Si-H stretching vibrations. The small band appearing around 1700 cm⁻¹ is attributed to carbonyl (C=O) groups. Absorptions in the regions of 1640–1620 and 1560–1400 cm⁻¹ are due to the C=C stretching vibrations in the aromatic ring. However, 1250–1000 cm⁻¹ is typically attributed to the C–O band in aromatic ethers, esters, phenols, carboxylic acids and alcohols. The bands in 900–700 cm⁻¹ show the C–H out of plane which is a characteristic of the aromatic substitution pattern.

3.2. Plate test

Preliminary plate assessments revealed that *Klebsiella* spp. could remove the red dye of Carmoisine from YTS+C after a time–space between 72 h and less than 96 h of cultivation (Figure 1). Moreover, spectrophotometry showed that no Carmoisine was detected in samples obtained from the treated cells picked up from the dye-removed plate (Figure 1). However, a Carmoisine peak at λmax = 515 nm appeared in the spectrum of Met-Sal+C,50ppm. In addition, thin layer chromatography exhibited two fluorescent spots for the sample of treated cells. This refutes the possibility of absorption and/or accumulation of Carmoisine into *Klebsiella* spp. cells but reinforces the hypothesis that bacteria could degrade Carmoisine into its aromatic amine constituents. Our previous study on the biological degradation of Carmoisine using *Saccharomyces cerevisiae* ATCC 9763 [29] indicated that this yeast required 4 days to completely remove Carmoisine from YPD+C (a medium similar to YTS+C once tryptone and sucrose were replaced by peptone and dextrose, respectively). Since the disaccharide such as sucrose needs to be hydrolyzed to monosaccharide in order to enter into a catabolic pathway [38], microorganisms require more time to grow on a sucrose-containing medium in comparison with a dextrose-containing medium under the same conditions. However, *Klebsiella* spp. cells could remove the Carmoisine from YTS+C at a rate higher than that achieved by *Saccharomyces* cells on YPD+C. Therefore,
it can be asserted that Klebsiella spp. exhibited greater potential for Carmoisine degradation in comparison with Saccharomyces.

3.3. Carmoisine debilitation in suspended anaerobic process

Klebsiella spp. cells in suspended form were used in anaerobic reduction of Carmoisine. Spectrophotometry measurements depicted as the debilitation of dye versus time (Figure 2) show that 98.2% of initial dye was debilitated during 56 h of incubation. In the same time period, sucrose was consumed by the cells, thus causing the sucrose concentration to decrease to 1.14% from an initial level of 2%. After 56 h of incubation, dye depletion fluctuated and reached 96.9% within 75 h of incubation. These fluctuations may be due to the slightly reverse reactions of accumulated aromatic amines caused by Carmoisine cleavage. The main kinetic parameters were determined as $K_S = 12.4$ g/l, $\mu_{\text{max}} = 0.021$ h$^{-1}$ and $Y = 0.056$ g/g, using the equations, 

$$r_{\text{SU}} = (kX/S) \cdot (K_S + S)$$

and

$$\mu = Y(kS/K_{S+}) - k_d.$$  

In these equations, $r_{\text{SU}}$ is the rate of substrate concentration change due to utilisation (g substrate/l.h), $k$ shows the maximum specific substrate utilisation rate (g substrate/g biomass.h), $X$ indicates biomass concentration (g/l), $S$ stands for growth-limiting substrate concentration in solution (g/l), $K_S$ is the half-velocity constant defined as substrate concentration at one-half the maximum specific substrate utilisation rate, g/l, $\mu$ is the specific biomass growth rate (h$^{-1}$), $Y$ is the synthesis yield coefficient (g biomass/g substrate) equivalent to $(\mu_{\text{max}}/k)$, $\mu_{\text{max}}$ is the maximum specific bacterial growth rate (h$^{-1}$) and $k_d$ is the endogenous decay coefficient (h$^{-1}$). The kinetic coefficients are in the range presented for common wastewater treatments in the literature [39]. Thin layer chromatography showed that the dye spot disappeared on TLC after 48 h and fluorescent spots were illuminated under UV light, which confirm the biotransformation of Carmoisine into its aromatic amine constituents. In addition, biosorption tests indicated a lack of Carmoisine adsorbed on or accumulated in bacterial cell pellets.

Suspended anaerobic processes using different inoculation sizes were performed for the debilitation of Carmoisine. Spectrophotometry showed that ≃96% of initial Carmoisine reduced during 24 h and displayed 2 fluorescent spots on the TLC sheet when cells of 50 or 100 ml preculture were used in the process (data not given). However, the use of the 4.5 ml preculture resulted in a 98.2% decrease in the initial concentration of Carmoisine during 56 h, and after 48 h of incubation, one fluorescent spot appeared on the TLC sheet (Figure 2). Consequently, when the inoculation size increased ≃10–20 times, the rate of process increased only ≃2.2 times, which may not be economically efficient. Differentiation in substitutions on aromatic amine (AA) rings produced in Carmoisine debilitation studies by

Figure 1. Display of visual changes in the solid medium of YTS + C streaked with Klebsiella spp. after (a) 24 h and (b) 72 h of incubation at 30°C. Spectrum of Met-Sal + C,50 ppm and supernatant of treated cells collected from the dye-removed YTS + C plate. The inset shows the TLC sheet under visible and UV lights; spots corresponding to n-butanol extracts of Met-Sal + C,50 ppm and supernatant of treated cells from left to right.
various inoculation sizes led to the appearance of fluorescent AA spots at one or two retention factors on the TLC sheet.

The metabolites scraped from the two fluorescent bands on TLC were analysed by LC–MS (Figure 3). The LC–MS spectrum of YS+C,50ppm medium revealed two major ion peaks at \(m/z=468\) and \(m/z=515\) indicating the Disodium 4-hydroxy-2-[(E)-(4-sulfonato-1-naphthyl) diazenyl]benzene-1-sulfonate or Disodium 4-hydroxy-2-[(E)-(4-sulfonato-1-phenyl) diazenyl]naphtalene-1-sulfonate and disodium 4-hydroxy-2-[(E)-(4-sulfonato-1-naphthyl)diazenyl]naphthalene-1-sulfonate, respectively. These compounds appeared as an adduct of the type \([M + OH]^−\) during initial ionisation [40]. Moreover, the \(m/z=147\) and \(m/z=227\) on LC–MS spectra corresponding to two spots appeared on the TLC of the Carmoisine-debilitated sample obtained after 27 h incubation of YS+C,50ppm medium with cells of 100 ml preculture. The \(m/z=147\) can be attributed to 4-Amino-1-naphthalene sulphonic acid sodium salt or 3-amino-4-naphthol-1- sulphonic acid sodium salt whose amine group appears as \(\text{NH}_2^+\) in solution and the \(\text{SO}_3\text{Na}\) branch is broken during ionisation in mass analysis. Therefore, \(m/z=227\) may be assigned to 4-Amino-1-naphthalene sulphonyl acid sodium salt whose \(\text{SO}_3\text{Na}\) branch is broken during ionisation in mass analysis. It is worth noting that fragile sulphate groups may be broken during the fragmentation in LC–MS analysis, and, therefore, the sulphate mass becomes lost in the resulting \(m/z\) [41,42]. In conclusion, the findings of the LC–MS analysis show that Klebsiella spp. had the potential to cleave the azo bond in the Carmoisine molecule and convert Carmoisine into its constituent aromatic amines.

### 3.4. Formation of the bed of AC-adhered bacteria

The number of viable cells was determined for preculure suspension used for the inoculation of a 17 g adsorbent and also for the unit mass of each adsorbent during the incubation with bacterial culture. Accordingly, the percent of viable cells (\(P_{\text{via}}\)) on adsorbents in relation to those present at the initial time of incubation was calculated by Equation (3):

\[
P_{\text{via}} = \frac{\text{CFU/unit mass of adsorbent}}{\text{Preculture volume} \times \left(\frac{\text{CFU/unit volume of pre-culture}}{\text{initial mass of adsorbent}}\right)} \times 100,
\]

where CFU means colony-forming units, the unit mass of adsorbent was 1 g, the pre culture volume was the size (4.5, 50 and 100 ml) of bacterial culture whose cell mass was used for inoculation, the unit volume of pre-culture was 1 ml, and the initial mass of the adsorbent was 17 g. A graph of \(P_{\text{via}}\) versus time in Figure 4 revealed that \(~40\%\) and \(~19\%\) of viable cells remained on granular and rod-shaped ACs, respectively, after three days of incubation where the bed was inoculated with biomass of 4.5 ml pre-culture. However, when biomass of higher amounts of pre-culture (50 ml or 100 ml) was used for the inoculation of the bed, \(P_{\text{via}}\) was less than 2% for both types of bed (granular and rod-shaped). This could be due to nutrient deficiency occurring in the presence of high cell density. A stable population between the third and fifth day of incubation was
observed and then a slight decline in cell counts revealed an endogenous decay process due to the aging of culture. However, Figure 4 shows that \( P_{\text{via}} \) was greater for the bed of granular ACs in comparison with rod-shaped ACs during the formation of the bacterial bed. This is due to the higher porosity of granular-activated carbons expressed in terms of surface area, pore volume and pore area in Section 3.1.

Scanning electron microscopy images (Figure 5) show the colonization and growing of bacteria on AC particles inoculated with cells harvested from the 4.5 ml preculture. From the fifth day of incubation, bacteria showed noticeable intertwining characteristics indicating biofilm formation. The confluence of bacteria extended in the following days so that most of the area of the AC surfaces was covered by biofilms by the tenth day of incubation.

Confocal microscopy (Figure 6) confirmed the attachment of bacteria on the surface of the AC particles. Moreover, fluorescent stain SYBR\textsuperscript{®} Green master mix could target the DNA of bacteria over the ACs without staining the background, which highly facilitates the discrimination of bacteria from the background. Kjellerup et al. [43] reported the higher

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**Figure 3.** LC–MS spectra (a) YS + C,50 ppm medium and the (b) upper and (c) lower spots scraped from the TLC sheet corresponding to sample obtained after 27 h incubation of YS + C,50 ppm medium with cells of 100 ml preculture, respectively. The TLC sheet shows the upper (b) and lower (c) fluorescent spots.
accuracy of the SybrGreen-CLSM imaging technique for the investigation of biofilms on AC particles in comparison with the SEM method. This is noteworthy since in SEM imaging, precipitates from media or sediments in AC particles can make it difficult to distinguish bacteria from the background. Moreover, high magnification is necessary for visualising the bacteria in SEM, which limits fields of view, whereas when using the SybrGreen-CLSM imaging technique, the entire particle is displayed, while bacteria remain visible. However, other fluorescent stains such as Hoescht 342 [44], DAPI or PNA-FISH [43] could not provide a qualified discerning display of bacteria on activated carbon particles.

Performance of the debilitation process was investigated using 5- and 10-day beds of granular and rod-shaped activated carbons inoculated with different amounts of cell suspension. In order to abbreviate the conditions used in each process, G4.5, G50, G100, R4.5, R50 and R100 were used to display the bed of granular (G) and rod-shaped (R) activated carbons inoculated with cells collected from 4.5, 50 and 100 ml of cell suspensions, respectively. TLC analyses (Figure 7) revealed the gradual disappearing of the Carmoisine spot and intensification of fluorescent spots during the process. This test essentially indicates the biotransformation of Carmoisine to aromatic amines via cell-attached beds, which was further investigated in biotransformation studies through HPLC analyses discussed in the following section. Figure 7 also shows that the debilitation of Carmoisine via 5-day beds led to one fluorescent spot on the TLC sheet, while 10-day beds led to two fluorescent spots. This may be due to differentiation in substitutions on aromatic amine rings produced in Carmoisine debilitation studies. Furthermore, Table 1 revealed that 10-day beds had higher rates but lower efficiencies in dye debilitation processes in comparison with 5-day beds. This may be due to the fact that the main fraction of bacteria was lysed during the 10-day period, resulting in a release of enzymes into the liquid phase which enjoyed more accessibility to Carmoisine. However, the enzymes in the liquid phase are more likely to be denatured and exhibit loss of activity potential, while more viable cells on the 5-day bed render

Figure 4. Percent of viable cells of Klebsiella spp. on granular (G) and rod-shaped (R) activated carbons during static incubation in YTS at 30°C used to form the bed of AC-adhered bacteria. Different sizes of preculture were used to inoculate the AC beds.

Figure 5. SEM micrographs showing the porous structure of (a) granular and (f) rod-shaped ACs used for bed formation as well as the bacteria growing over (b–e) granular and (g–j) rod-shaped ACs after 3, 5, 7 and 10 days of incubation in YTS at 30°C from left to right, respectively.
more intact enzymes but with less accessibility. In addition, Table 1 shows that an increase in cell biomass inoculated on AC beds could not significantly improve the rate or efficiency of Carmoisine debilitation. This is due to the fact that a great percentage of cells were lost during the AC-bed formations due to scarce nutrition (Figure 4). Moreover, as Table 1 shows, on average, beds formed of granular ACs exhibited a longer effective time leading to a lower effective rate in comparison with rod-shaped ACs in Carmoisine debilitation. But in this comparison, the higher effective efficiencies were achieved by GAC beds. Due to more viable cells which grew on GAC particles in comparison with RAC particles, as shown in Figure 4, greater efficiencies were obtained in debilitations of Carmoisine by GAC beds. However, the lower rate of GAC beds is attributed to mass transfer limitations into the pores, leading to reduced bacterial accessibility to Carmoisine. Since the bacteria mainly colonise in pores, the higher pore volume and pore area of GAC particles contributed to the reduced availability of dye to bacteria on GACs.

Furthermore, spectrophotometry measurements revealed that the cells of 4.5 ml pre-culture achieved ∼98% Carmoisine debilitation efficiency during ∼56 h in a suspended anaerobic process (Figure 2), whereas these cells attained this efficiency in only 6–8 h in a BAC process (Table 1). Therefore, an increased rate of process was achieved by the use of the AC-attached cells in the Carmoisine debilitation process, which can be considered an advantage for the use of immobilised systems in establishing the processes [10–12].

3.5. Biotransformation studies
HPLC chromatograms shown in Figure 8 exhibited peaks at similar retention times with slight shifts. The peak areas were used for better recognition. The main peaks appeared in two sets of triplicate and duplicate, specified with circles and squares, respectively. The first peak in the triplicate set which appeared similarly in the samples of YS, YS+C,50 ppm, YS+C,100 ppm, YS+A,50 ppm and YS+A,100 ppm (Figure 8(a–e)) is attributed to YS components. This peak appeared in the area of 1105 mV s in YS medium, which decreased to ∼544–604 mV s in YS media containing dye or amine (Table 2). Intermolecular forces among the YS components and dye or amine cause the peak to shrink. This peak appears in a chromatogram of the sample prepared at the end of the anaerobic-suspended process (Figure 8(f)) with an area of ∼418 mV s (Table 2), showing the consumption of the YS components during the microbial process.
The second peak of the triplicate set in Figure 8(a–e) is assigned to YS components, dye and amine. This peak increases the area with an increase in the concentration of dye or amine (Table 2) in YS medium. Therefore, a significant increase in the area of the second peak of the triplicate set displayed by the end sample of the anaerobic-suspended process (Figure 8(f) and Table 2) in comparison with the sample of YS+C,50ppm medium can be attributed to the presence of amine in the end sample due to a lack of dye in the samples, according to TLC and spectrophotometry data (Figure 2). Therefore, the biotransformation of Carmoisine to aromatic amine by Klebsiella spp. is confirmed.

The second peak of the duplicate set in Figure 8(a–e) may be assigned to YS components, dye and amine. This peak noticeably increased with area in the presence of dye or amine. Therefore, the area of the first peak of the duplicate set in Figure 8(f) and Table 2 confirmed the presence of aromatic amines in sample at the end of the anaerobic-suspended process. Loss of Carmoisine was also demonstrated via TLC and spectrophotometry observations (Figure 2). Accordingly, the biotransformation of Carmoisine into its amine constituents during the process with Klebsiella spp. was again confirmed.

The third peak of the triplicate set in Figure 8(a–e) may be ascribed to YS components, dye and amine. This peak noticeably increased with area in the presence of dye or amine. Therefore, the area of the first peak of the duplicate set in Figure 8(f) and Table 2 confirmed the presence of aromatic amines in sample at the end of the anaerobic-suspended process. Loss of Carmoisine was also demonstrated via TLC and spectrophotometry observations (Figure 2). Accordingly, the biotransformation of Carmoisine into its amine constituents during the process with Klebsiella spp. was again confirmed.

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Figure 8. HPLC chromatogram of metabolites extracted from (a) YS, (b) YS + C, 50 ppm (c) YS + C, 100 ppm (d) YS + A, 50 ppm (e) YS + A, 100 ppm media and processed media with (f) suspended cells, (g) GAC-attached cells, (h) GAC control, (i) RAC-attached cells and (j) RAC control.
The area of this peak decreased in sample at the end of the anaerobic-suspended process due to the consumption of YS medium components during the microbial process.

Chromatograms in Figure 8(g–j) show that the first peak of the triplicate set appeared with a reduced area (Table 2) expressing the consumption and adsorption of YS components by AC-attached cells in the anaerobic process and ACs in control experiments, respectively. The second and third peaks of the triplicate set appearing in Figure 8(g,i) confirmed that aromatic amines were present in samples prepared at the end of the process with attached cells, which is in accordance with spectrophotometry and TLC outcomes (Table 1 and Figure 7). The small area of these peaks is due to some adsorption by ACs. Moreover, the second and third peaks of the triplicate set are absent due to the adsorption of dye by ACs. In addition, the first peak of the duplicate set appeared on chromatograms in Figure 8(g–j), indicating the presence of aromatic amines and dye in samples prepared at the end of the anaerobic process with AC-attached cells and control experiments with ACs, respectively. This conclusion can be inferred along with spectrophotometry and TLC results (Table 1 and Figure 7). The second peak of the duplicate set is absent in chromatograms in Figure 8(g–j), showing the consumption and adsorption of YS components by AC-attached cells in the anaerobic process and ACs in control experiments, respectively.

It can, therefore, be concluded that *Klebsiella* spp. has the potential to biotransform Carmoisine into its constituent aromatic amines in both forms of suspended and attached growth processes.

### 3.6. Sustainability of AC-adhered bacteria in repeated processes

Bacterial beds prepared during five days of inoculation with *Klebsiella* spp. cells collected from the 4.5 ml precul-ture were used for Carmoisine debilitation in several repeated batches. The percent of viable cells ($P_{\text{via}}$) on the 5-day beds was estimated via Equation (3) before use in the Carmoisine debilitation process and at the end of each batch. Table 3 shows that $P_{\text{via}}$ considerably reduced on both GAC and RAC beds at the end of the first batch and a second decrease in $P_{\text{via}}$ on the GAC bed was observed at the end of the second batch. After a relatively steady state of $P_{\text{via}}$ on the beds, a sudden decrease occurred at the end of the second batch. After the steady state of $P_{\text{via}}$ on the beds, a sudden decrease occurred at the end of the fifth batch. Then, at the end of the sixth batch, a dramatic increase in $P_{\text{via}}$ on the beds was observed due to the incubation of the beds for 48 h in YTS medium. Also, $P_{\text{via}}$ increased slightly at the end of the seventh batch. The percentage of

### Table 2. Retention time and area of peaks detected in HPLC chromatograms.

| Table 2 | Retention time and area of peaks detected in HPLC chromatograms. |
|---------|------------------------------------------------------------------|
| **YS** | **YS+C, 50 ppm YS+C, 100 ppm YS+A, 50 ppm YS+A, 100 ppm Suspension cell GAC a-Cell GAC RAC b-Cell RAC Triplicate set First peak Retention time (min) 10.58 10.03 10.76 11.55 10.5 10.6 10.71 10.76 10.3 10.53 Area (mV s) 1105.52 544.95 596.49 550.28 604.02 418.43 102.21 58.03 97.37 68.51 Second peak Retention time (min) 12.38 11.16 12.1 12.6 11.8 12.1 12.03 11.33 11.7 11.33 Area (mV s) 493.98 616.37 714.83 656.46 749.44 1705.71 94.74 11.71 197.76 11.76 Third peak Retention time (min) 13.11 12.28 13.35 14.08 13.26 13.26 13.26 13.33 14.08 13.33 Area (mV s) 521.10 114.63 114.09 175.80 457.62 1668.86 29.84 12.03 73.50 45.93 Duplicate set First peak Retention time (min) 18.93 16.66 17.9 18.71 17.5 18.13 17.61 17.01 16.83 17.8 Area (mV s) 50.92 606.46 530.56 547.94 530.56 390.29 342.67 300.19 290.29 17.8 Area (mV s) 478.70 500.42 497.70 539.74 539.74 497.70 539.74 497.70 497.70 17.8 Area (mV s) 478.70 500.42 497.70 539.74 539.74 497.70 539.74 497.70 497.70 17.8 |
Carmoisine debilitation during 6 h of incubation remained between ∼95% and ∼99% in various batches despite reduced $P_{\text{via}}$ in some batches (Table 3). In fact, $P_{\text{via}}$ decreased due to the lysis of cells and this resulted in the release of enzyme content out of cells and enzyme adsorption on beds, which, in turn, maintained the efficiency of Carmoisine degradation. Moreover, TLC analysis showed the biotransformation of Carmoisine into aromatic amines in all batches (Supplementary Figure S2). Therefore, consecutive batch experiments demonstrated the reusability potential of AC-attached cells for Carmoisine debilitation, which is expected to act as an advantage of the immobilised matrix to improve the economic performance of the process [13].

4. Conclusions

This study demonstrates the feasibility of using Klebsiella spp. for Carmoisine debilitation in both suspended and adhered activated carbon particles. The biological activated carbon process could improve the rate of Carmoisine debilitation in comparison with suspended processes and provide reusability of bacterial cells in consecutive batches. Different chemical analyses revealed that biodegradation played a major role in the decolorisation of Carmoisine. The size of inoculation, pore volume and pore area of activated carbon used for biological bed formations as well as the bed age are the most important factors determining the rate and efficiency of the BAC process. It can be concluded from this work that activated carbon as the immobilisation medium provided macro pores for cell growth, resulting in an increased biotransformation of dye in terms of rate and efficiency.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Table 3. Percent of viable cells of Klebsiella spp. on granular (G) and rod-shaped (R) activated carbons at the end of each batch of Carmoisine debilitation.

| Batch number | Five-day bed before use in the Carmoisine debilitation process | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|--------------|---------------------------------------------------------------|---|---|---|---|---|---|---|
| Percent of viable cells | G | 35.95 ± 0.71 | 26.32 ± 0.52 | 14.94 ± 0.29 | 15.74 ± 0.31 | 13.35 ± 0.26 | 1.39 ± 0.02 | 13.18 ± 0.26 | 16.80 ± 0.33 |
| | R | 17.00 ± 0.34 | 9.43 ± 0.18 | 11.24 ± 0.22 | 7.92 ± 0.15 | 9.15 ± 0.18 | 4.22 ± 0.08 | 7.13 ± 0.14 | 11.54 ± 0.23 |
| Percent of Carmoisine debilitation | G | – | 98.72 ± 1.86 | 99.98 ± 0.89 | 99.79 ± 0.13 | 95.16 ± 3.27 | 98.96 ± 2.68 | 97.15 ± 1.27 | 96.11 ± 0.73 |
| | R | – | 95.69 ± 1.49 | 99.96 ± 3.01 | 99.13 ± 0.58 | 96.23 ± 2.05 | 97.8 ± 1.11 | 98.17 ± 0.26 | 97.90 ± 0.19 |

Note: Five-day granular (G) and rod-shaped (R) beds inoculated with Klebsiella spp. cells of 4.5 ml preculture for Carmoisine debilitation. Percent of Carmoisine debilitation was calculated for each batch after 6 h of incubation.

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Availability of data and materials

The bacterial strain Klebsiella quasipneumoniae GT7 (NCBI accession number: KY357316) was isolated from the expired liquid date sugar, and identified as Klebsiella quasipneumoniae according to the morphological characteristics, biochemical tests and 16S rRNA sequence analysis. The strain (PTCC No: 1938) was deposited in the Persian Type Culture Collection, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. Requests to access this strain should be directed to Tayebe Bagheri Lotfabad (bagheril@nigeb.ac.ir).

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