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Biotransformation of ethyl 2-(2′-nitrophenoxy)acetate to benzohydroxamic acid (D-DIBOA) by *Escherichia coli*

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ABSTRACT

Benzohydroxamic acids, such as DIBOA, exhibit interesting biological properties (herbicidal, fungicidal and bactericidal). Recently, the synthesis of DIBOA has been simplified to only two steps. This paper explores the possibility of replacing the second stage in the chemical synthesis of D-DIBOA by a biotransformation using a strain of *Escherichia coli* and a strain of *Serratia marcescens*. Biotransformation experiments were carried out for both strains in the presence of different concentrations (0.25, 0.5 and 1 mg/mL) of the precursor (*ethyl 2-(2′-nitrophenoxy)acetate*) under aerobic and anaerobic conditions. Both strains tolerated the concentrations of precursor investigated here. Higher biotransformation yields were reached for *E. coli* under aerobic conditions. The UV/vis spectra and $^{1}H/^{13}C$ spectroscopic data obtained from HPLC-DAD and NMR, respectively, for the compounds obtained in the biotransformation reaction confirmed the presence of D-DIBOA in cultures of *E. coli*. The maximum yields were obtained in experiments supplemented with 0.5 mg/mL of precursor and these were $20.14 \pm 1.87\%$ under aerobic conditions and $8.17 \pm 0.94\%$ under anaerobic conditions.

1. Introduction

The isolation, structural elucidation, and synthesis of natural products constitute the main source of new molecules with biological activity [1]. Compounds with a (2H)-1,4-benzoxazin-3(4H)-one skeleton (benzohydroxamic acids) have attracted the attention of phytochemistry researchers since 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (known as D-DIBOA, see Fig. 1) and 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxacin-3(4H)-one (known as D-DIMBOA, see Fig. 1) were isolated from plants belonging to the Poaceae family [2,3]. These compounds exhibit interesting biological profiles that include phytotoxic, antimicrobial, antifeedant, antifungal, and insecticidal properties [4,5]. A number of syntheses of benzohydroxamic acids have been reported in the literature [6–9].

Recently, the synthesis of D-DIBOA has been simplified to only two steps (Fig. 2). The first step involves functionalization of the phenol by nucleophilic substitution to introduce the appropriate side chain (from ethyl bromoacetate) under mild conditions using nitrophenol as the starting material. The reaction product is *ethyl 2-(2′-nitrophenoxy)acetate* and this is obtained in yields up to 99%. The second step involves reduction of the nitro group followed by a cyclization involving intramolecular addition/removal of the ethyl chain from the ester. This step is heterogeneously catalysed by a Pd/C catalyst and the conditions are critical due to the exothermic character of the reaction and the release of hydrogen [6].

One of the alternatives in the total chemical synthesis is the replacement of some stage of the synthesis by a process involving microbial action. This type of process is called biotransformation and can be defined as the modification of a chemical compound by a living organism. There are a wide range of products of secondary origin that can be metabolized and processed by enzymes or microbial cells. These products are interesting in terms of stereoselectivity, regiospecificity and conversion into derivatives, but also for the advantages related to the reaction conditions in which they occur. Such products are currently applied in the production of drugs and other substances of industrial interest.

The hypothesis suggested in this study is that it is possible to reduce the nitro group of *ethyl 2-(2′-nitrophenoxy)acetate* by a biotransformation that replaces the second stage of the synthesis described above. The biological reduction of nitro groups in a molecule analogous to *ethyl 2-(2′-nitrophenoxy)acetate*, namely trinitrotoluene (TNT), has been documented in several reports. The reductive transformation of TNT has been documented for many bacterial genera, e.g. *Serratia* [11], *Escherichia* [12], *Desulfovibrio* [13], *Clostridium* [14,15], and *Pseudomonas* [16]. Several of these publications indicate that transformation of the nitro group, in aerobic or anaerobic conditions, to an amine occurs through the

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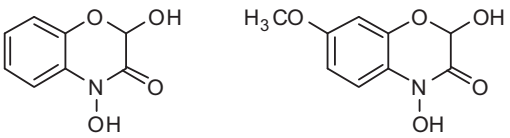

Fig. 1. Natural benzoxazinones. 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3-(4*H*)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3-(*4H*)-one (DIMBOA) [10].

formation of the corresponding hydroxylamine [12,15–17]. This kind of biotransformation is quite similar to the second stage of the process outlined in Fig. 2. This fact suggested that these microorganisms could be capable of carrying out this step.

The reduction of the nitro groups via hydroxylamines can be attributed to nitroreductases [18,19]. In the Enterobacteriaceae family, in addition to the species *Escherichia coli*, several enzymes with nitroreductase activity have been characterized in *Enterobacter cloacae* [20], particularly a xenobiotic reductase that reduces nitro groups on the ring to give the hydroxylamine derivative of TNT [21].

The aim of the work described here was to explore the possibility of replacing the second step in the chemical synthesis of D-DIBOA by a biotransformation using a strain of *E. coli* and a strain of *Serratia marcescens*.

2. Materials and methods

2.1. Microorganisms and chemicals

The strain *E. coli* JM109 (ATCC 53323)/pGEM4Z (Promega) was obtained from ATCC. *S. marcescens* strain N2 belongs to the microbial collection of the Metal Biotechnology Laboratory, Biology Faculty, Havana University. This strain was isolated from a lateritic deposit from Moa, Holguin (Cuba) [22].

The starting compound used to study the biotransformation was *ethyl 2-(2′-nitrophenoxy)acetate*, which is henceforth referred to as the precursor.

Chemical standards of the precursor and D-DIBOA were synthesized from *2-nitrophenol* as described by Macías et al. [6]. The reagents were purchased from Sigma–Aldrich and the culture media from Panreac, FLUKA and Cultimed. All organic solvents were HPLC grade.

2.2. Media

The inoculums required for experimentation were obtained by seeding bacteria from conservation strains in Luria-Bertani broth (LB). The inoculum/medium ratio was 1:10 (v/v) and the bacteria were grown overnight at 30 °C in Erlenmeyer flasks. These pre-inoculums were used to inoculate (10%, v/v) test systems for both aerobic and anaerobic conditions.

In aerobic experiments LB broth was inoculated (100 mL final volume) in Erlenmeyer flasks that were kept in an orbital incubator at 150 rpm and 30 °C. For anaerobic cultures LB broth was inoculated (50 mL final volume) in sealed vials and incubation was carried out at 30 °C under static conditions.

2.3. Biotransformation experiments

Strains used in this study were selected for their ability to reduce nitro groups of TNT as this indicates that they may be capable of performing the reduction of the nitro group in the precursor.

The transformation of the precursor was initiated by adding 1 mL of stock precursor solutions diluted in methanol (MeOH) for the aerobic culture, or 0.5 mL for the anaerobic culture, to achieve concentrations of 0.25, 0.5 and 1.0 mg of precursor/mL. The inoculation was performed from cultures of strains *E. coli* and *S. marcescens* in the exponential phase of growth (10%, v/v). During the incubation, 1 mL and 0.5 mL samples for aerobic and anaerobic conditions, respectively, were withdrawn at 0, 9 and 24 h. The samples were harvested by centrifugation for 10 min at 10,509 × *g* and the supernatants were filtered through 0.22 μm nylon filters prior to analysis by high-performance liquid chromatography (HPLC).

Two kinds of control experiments were performed: a biotic control, i.e. a bacterial culture without addition of precursor, and several abiotic controls, i.e. culture medium supplemented with the same concentrations of precursor tested without inoculum. The controls were kept under the same conditions of agitation and temperature as for the related experiments.

A biotransformation experiment was subsequently carried out in order to confirm the identities of the reaction products. In this process, *E. coli* was cultivated in the presence of 0.75 mg/mL of precursor. The culture was monitored by Thin Layer Chromatography (TLC) during 48 h. After this time, the bacterial culture was centrifuged for 10 min at 10,509 × g. The supernatant was withdrawn and the pellet was resuspended in distilled water and centrifuged again. The total supernatant from both centrifugations (free of cells) was extracted ten times with ethyl acetate (EtOAc) at room temperature. The solvent was removed from the organic phase on a rotary evaporator under reduced pressure at 35 °C. The resulting crude extract was chromatographed on silica gel columns, using hexane/EtOAc with increasing polarity (from 1:4 to 1:1 (v/v)).

2.4. Analytical methods

2.4.1. Spectrophotometry

Bacterial growth experiments were monitored by following the increase in turbidity of the culture at 600 nm using a UV spectrophotometer (Model HITACHI U-2001). The bacterial population was represented by counting colony-forming units per mL (CFU/mL); this measure was correlated with turbidity by the McFarland scale [23]. All cultures were carried out in duplicate; the values presented are the mean of two experiments.

2.4.2. High-performance liquid chromatography (HPLC)

Reverse-phase HPLC analysis was performed on a Merck HITACHI HPLC system equipped with an L-7100 LaChrom quaternary gradient pump, an L-7200 LaChrom autoinjector and an L-7455 LaChrom diode array detector (DAD). Data were collected and processed using a Merck HITACHI D-7000 HPLC data system. Instrumental conditions were Phenomenex® Gemini C18 (4.6 mm × 250 mm) reversed-phase column at 25 °C. Mobile phases were as follows: water: 1% AcOH (A) and MeOH:1% AcOH (B) at a flow rate of 1 mL/min. The injection volume was 15 μL. The following gradient was used for separation: at 0 min, 0% B; 7 min, 30% B; 30 min, 40% B; 35 min, 100% B.

Chromatogram analysis was carried out using HPLC System Manager software Version 4.1 with the diode array detector set at 253 nm by comparing the retention times (RT) and ultraviolet/visible spectra with those of the chemical standards.

The fractions eluted from the silica gel column were further purified by HPLC on semipreparative silica gel (Lichrospher Si-60, 10 μm) columns using a mixture of hexane/EtOAc (6:4, v/v).

2.4.3. Nuclear magnetic resonance (NMR)

^{1}H and ^{13}C NMR spectra were run on Varian INOVA-400 spectrometers. Chemical shifts are given in parts per million (ppm) with respect to the residual ^{1}H signal of deuterated chloroform ($CDCl_3$) (δ 7.25) and the ^{13}C signal of chloroform ($CHCl_3$) (δ 77.00).

2.4.4. Thin layer chromatography (TLC)

TLC was carried out on Alugram Sil G/UV254 plates (Merck) with a layer thickness of 0.25 mm with fluorescent indicator. Chromatography plates were developed by treatment with oleum prepared with a solution of sulphuric acid, water and acetic acid (1:4:20) (v/v) followed by heating at 150 °C. The sample spots were observed using a UV lamp at 254 or 360 nm.

2.5. Biotransformation yield

For quantitative analysis, stock solutions of each individual chemical standard were prepared by dissolving accurate amounts in LB broth with 1% MeOH (v/v). Working D-DIBOA standard solutions of 0.25, 0.125, 0.0625, 0.0313 mg/mL were obtained by dilution of the 0.5 mg/mL stock. These solutions were used to generate the external standard response calibration curves for subsequent measurements of D-DIBOA concentrations from bacteria culture samples. Hydroxamic acids could

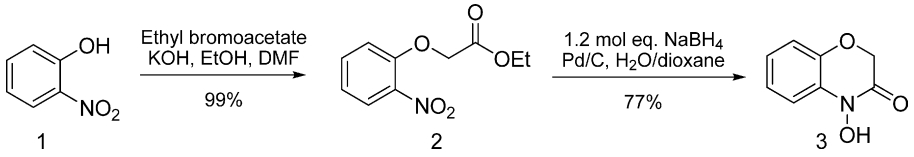

Fig. 2. Benzohydroxamic acid synthesis: (A) first step: synthesis of *ethyl 2-(2′-nitrophenoxy)acetate* (2) (the precursor) using nitrophenol (1) as starting material and (B) second step: reduction of the precursor nitro group to obtain D-DIBOA (3) [6].

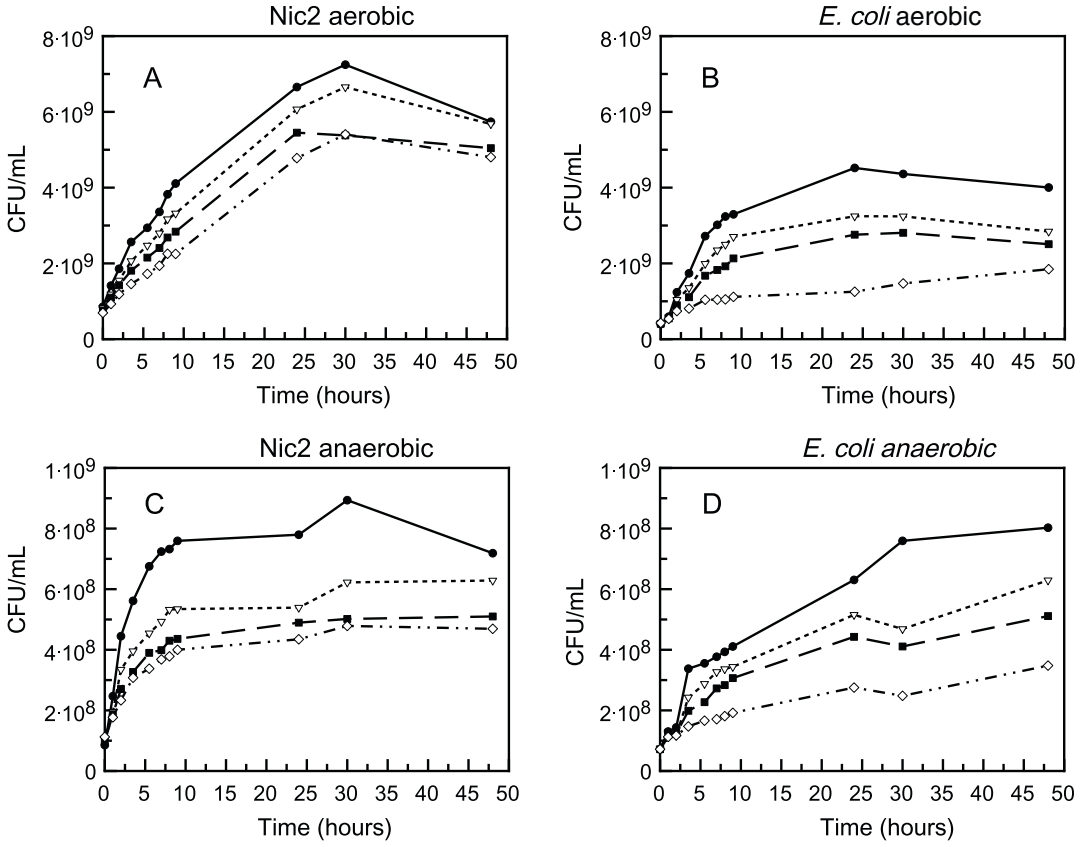

Fig. 3. Bacteria growth curves. Cultures in LB medium supplemented with increasing concentrations of precursor. (A) Nic2 and (B) *E. coli* in aerobic conditions; (C) Nic2 and (D) *E. coli* in anaerobic conditions. (—●—) 0 mg/mL control precursor; (···▽···) 0.25 mg/mL precursor; (—■—) 0.5 mg/mL precursor and (--◇--) 1.0 mg/mL precursor.

be good chelator of iron, then the quantification of D-DIBOA could be affected if iron complex is formed. For this reason, we determined the influence of the iron (III) concentration present in the medium to D-DIBOA through the examination of UV/vis spectra and the peaks areas of the chromatograms (data not shown). Finally, we can conclude that the iron content in LB broth does not affect the quantification of D-DIBOA in the culture conditions.

Data for biotransformation yields are presented as percentage of biotransformation, which was calculated thorough the molar relation of D-DIBOA at 24 h and the initial quantity of precursor supplemented in each experiment.

3. Results

3.1. Growth curves and response to precursor

Tracking the growth of *S. marcescens* and *E. coli* in CFU/mL for 48 h allowed us to establish the tolerance of these strains to the precursor. Growth was observed in both cultures under aerobic and anaerobic conditions and this was affected by increasing the concentration of precursor. Concentrations above 1 mg/mL were tested but these proved to be inhibitory for cell growth (data not shown), hence biotransformation experiments were conducted at concentrations below this level.

In general, it appears that the aerobic growth of both strains is similar, with the cell population showing maximum decreases as the concentration of precursor in the medium was increased. However, the absolute CFU/mL values achieved are significantly higher for *S. marcescens* than for *E. coli* (Fig. 3(A) and (B)). Under anaerobic conditions the behaviour of the two strains was very different, with *S. marcescens* practically reaching a stationary phase at 10 h while *E. coli* peaks are reached at the end of the experiment. In any case, the absolute values for both strains are 10 times smaller than under aerobic conditions.

The different behaviours observed are due to the different natures of the two strains, the different culture conditions tested (aerobic/anaerobic) and the possible inhibitory action exerted by the products that appear in the medium due to microbial metabolism.

3.2. Identification of compounds by HPLC

The samples were monitored at 253 nm and their component/s identified by diode array detector. Three chromatographic peaks were observed in chromatograms of samples from the cultivation of *E. coli* and *S. marcescens* at 9 and 24 h: compound I (Fig. 4(A)) and compound II (Fig 4(B)) appear at RT of 20.41 and 20.74 ± 0.04 min, respectively, and compound III (Fig. 4(A) and (B)) at 24.69 ± 0.04 min.

Compounds I and II were identified as D-DIBOA as they eluted very close to the reference standard (RT 20.43 min).

In addition, the UV/vis spectra of compounds I and II showed a high correlation to that of the D-DIBOA reference under both sets of conditions, with a correlation coefficient of 0.9999 for compound I and 0.9342 for compound II (Fig. 5(A) and (B), respectively).

The presence of compounds I and II was not evidenced in the chromatograms corresponding to biotic and abiotic controls, thus demonstrating that production of these compounds is the result of the transformation of the precursor by bacteria.

Compound III eluted more slowly than the precursor (24.51 ± 0.16 min) and the UV/vis spectrum had a high correlation coefficient with the precursor (0.9962) (Fig. 5(C)). This compound was detected in all of the analyzed samples from both cultures and also the abiotic controls, in both cases with the precursor, but it was not present in the biotic control. These findings seem to indicate that this compound is formed by chemical

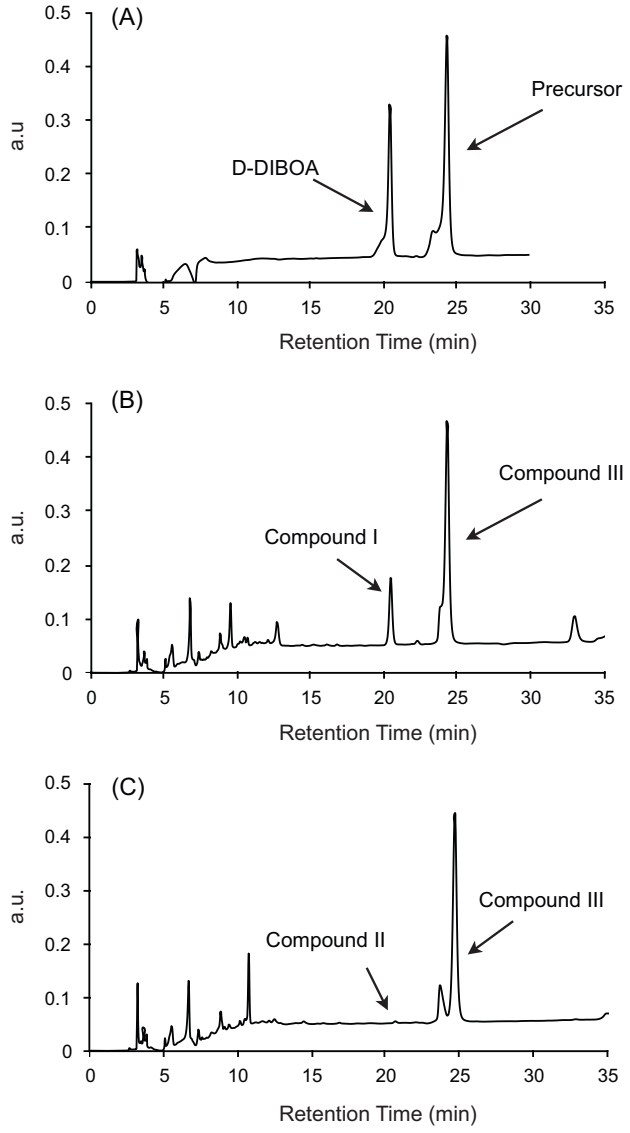

Fig. 4. *E. coli* and *S. marcescens* chromatograms. (A) Chromatographic peak areas for reference compounds, D-DIBOA and precursor (B) chromatographic peak areas for compounds in *E. coli* culture (C) chromatographic peak areas for compounds in *S. marcescens* culture.

degradation of the precursor. However, the structure of compound III remains unknown.

The graphs shown in Fig. 6 represent the chromatographic peak areas obtained for the precursor and compounds I and II under the different conditions tested for *E. coli* and *S. marcescens* cultures. It can be seen that the precursor concentration decreases significantly at 9 h (Fig. 6(A)) and reaches levels below the limit of detection at 24 h – except for *S. marcescens* cultures under anaerobic conditions, where the concentration is almost constant between 9 and 24 h (Fig. 6(C)). In the case of *E. coli*, the decrease in the concentration of precursor during the experiment can be correlated with the increase in the concentration of compound I, as can be seen by the peak areas for this compound in the chromatograms recorded at the two measurement times (Fig. 6(B) and (D)). However, this relationship is not observed in the case of compound II. In this case the disappearance of the precursor cannot be correlated with the appearance of compound II. Moreover, it is evident that *S. marcescens* under anaerobic conditions is not able to transform the precursor, which suggests some inhibitory effect for this compound on the metabolic activity of this strain in the absence of oxygen.

3.3. Analysis of metabolites in culture supernatants

Since the experiments with *E. coli* showed better biotransformation yields of the precursor, it was decided to analyze the metabolites in order to identify and characterize the compounds obtained in these experiments. To this end, a liquid–liquid extraction was carried out on the culture supernatant with ethyl acetate.

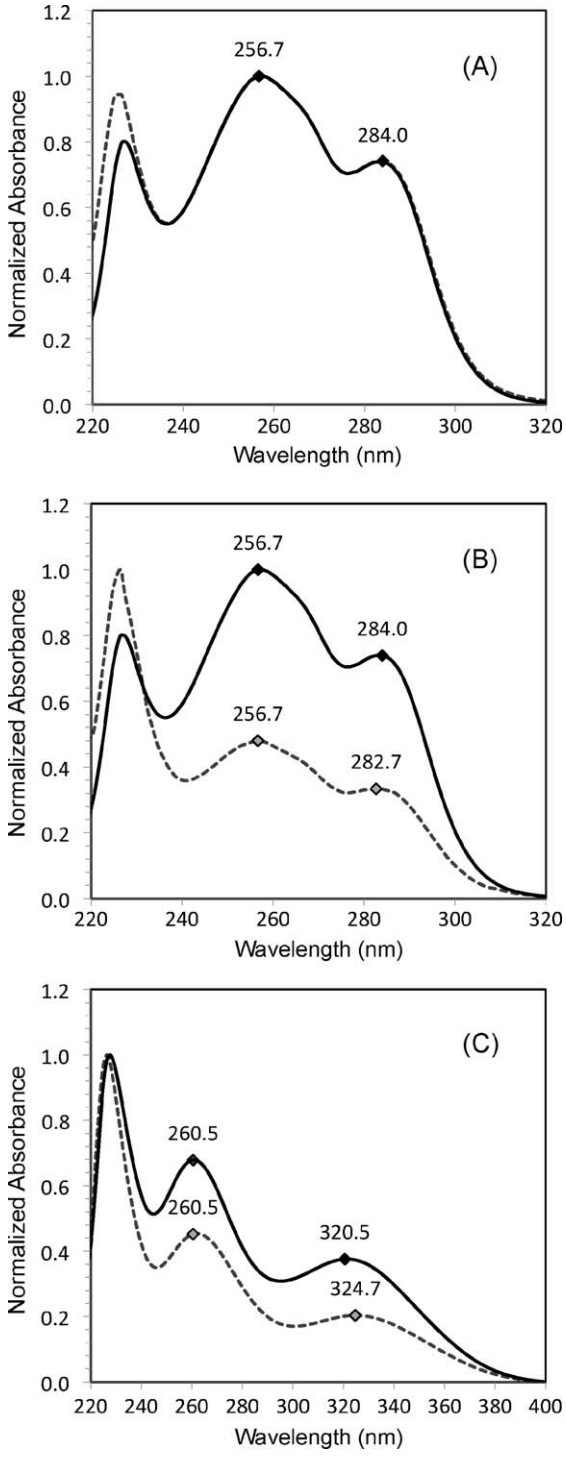

Fig. 5. UV–vis spectra of compounds present in the biotransformation experiments. Comparison of normalized UV–vis spectra of: (A) (—) D-DIBOA analytical standard and (– –) compound I from *E. coli* culture sample identified as D-DIBOA with a 0.9999 correlation coefficient. (B) (—) D-DIBOA analytical standard and (– –) compound II from Nic2 culture sample identified as D-DIBOA with a 0.9342 correlation coefficient. (C) (– –) compound III and (—) precursor analytical standard with a 0.9962 correlation coefficient.

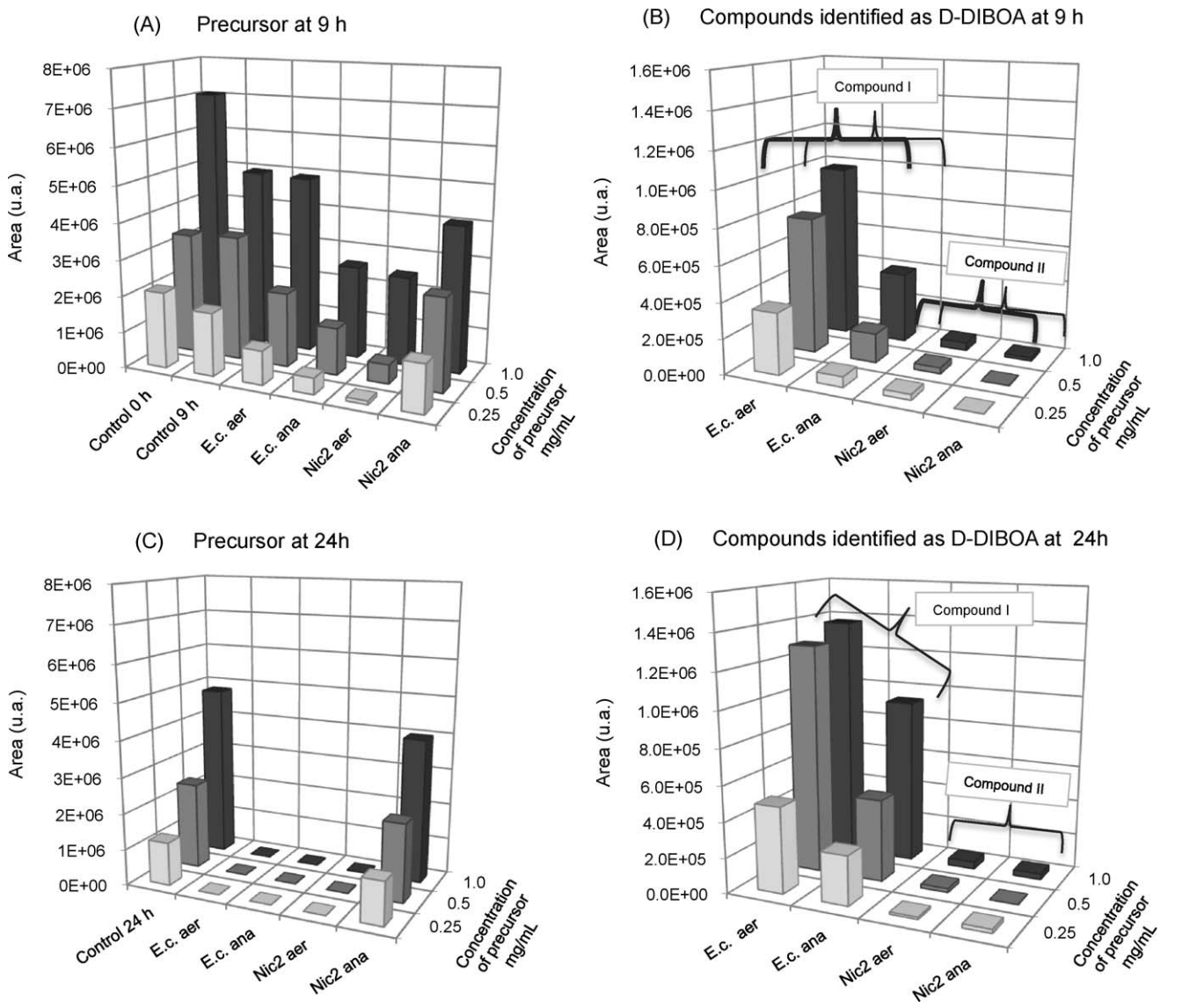

Fig. 6. Areas of precursor, compound I and compound II. Precursor areas (a.u.) in *E. coli* and Nic2 culture samples at: (A) 9 h and (C) 24 h. Compound I and II areas (a.u.) identified as D-DIBOA in *E. coli* and Nic2 culture samples respectively at: (B) 9 h and (D) 24 h. Precursor concentration [mg/mL]: 0.25, 0.5 and 1 mg/mL precursor concentration added at the start of the lag time. Culture bacteria conditions: experiments were carried out aerobically and anaerobically in both strains. Graph (A) represents the area corresponding to abiotic control at 0 and 9 h to facilitate comparison with the rest of the experiments.

The solvent was removed on a rotary evaporator to give 82.5 mg of extract (AcOEt-A). The extract (AcOEt-A) was separated into four fractions with hexane/AcOEt (6:4) and methanol and the following amounts were obtained: 1 (6.5 mg), 2 (6.3 mg), 3 (1.7 mg) and 4 (33.7 mg). These fractions were subsequently chromatographed by reverse phase HPLC (C-18 column). In these fractions, the only product identified as D-DIBOA on comparing the RT and UV/vis spectra (Fig. 4(A)) was detected in fraction 4 (33.7 mg). Furthermore, fraction 4 contained impurities and was therefore purified further on a silica gel column to give 8 mg of pure product.

The ^{1}H NMR (Fig. 7(B)) and ^{13}C NMR spectra of the pure product in $CDCl_3$ were identical to that of D-DIBOA (Fig. 7(A)) obtained using the procedure described by Macías et al. [6], which unequivocally confirms the structure.

The biotransformation yields obtained for *E. coli* are shown in Table 1. The best results were obtained under aerobic conditions in the experiment involving the addition of precursor at 0.5 mg/mL to give a yield of 20.14%. Under anaerobic conditions the yields were lower – between 8.17 and 9.19% for the three precursor concentrations tested.

Table 1
Biotransformation yield in *E. coli*. *E.coli* culture at 24 h in aerobic and anaerobic conditions. Precursor added to initial culture. The results are shown as mean ± Standard Deviation (S.D.).

Precursor concentration [mg/mL]	Biotransformation yield %	
	Aerobic	Anaerobic
0.25	11.2 ± 0.8	9.8 ± 0.8
0.5	20.1 ± 1.9	8.2 ± 0.9
1	10.8 ± 2.2	8.2 ± 0.6

4. Discussion

In tests with concentrations up to 0.5 mg/mL, the amount of compound I obtained was proportional to the initial concentration of precursor. At higher concentrations a further increase in biotransformation was not observed, a finding that can be attributed to inhibitory effects. Aerobic conditions gave better results in the biotransformation and this could be due in part to the fact that, during these trials, agitation was used and this helps mass transfer,

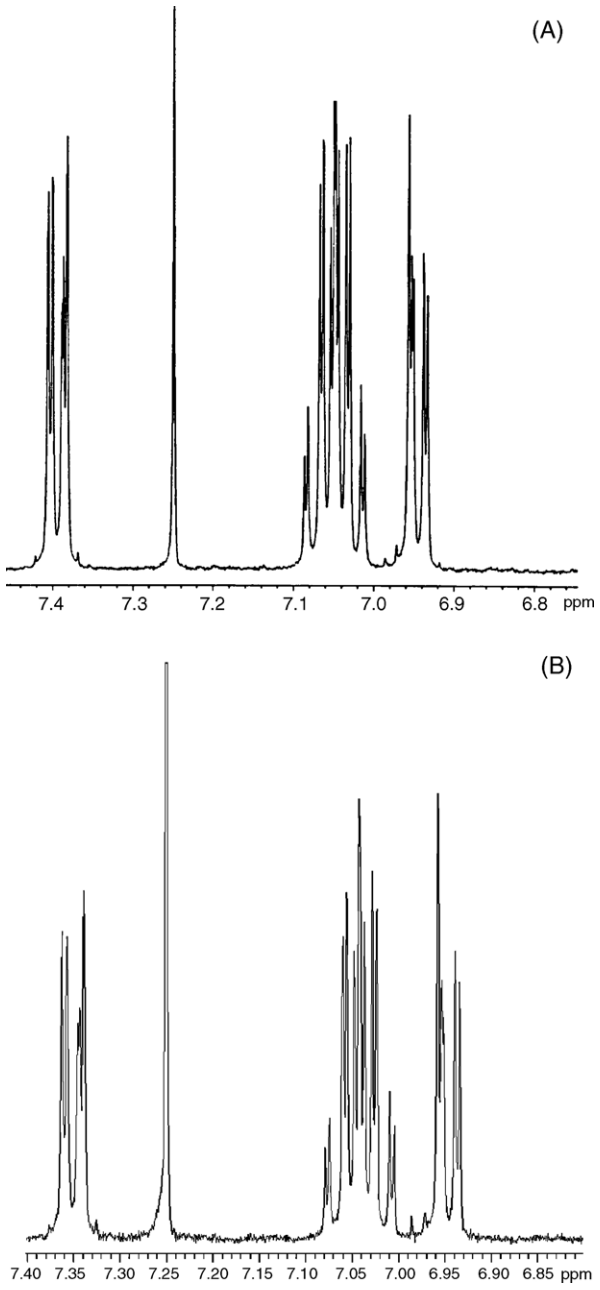

Fig. 7. ^{1}H NMR spectra of D-DIBOA. ^{1}H NMR spectra aromatic ring in ppm (Varian 400 spectrometer) of: (A) D-DIBOA from chemical synthesized and (B) D-DIBOA from *E. coli* transformation. Both samples were prepared in $CDCl_3$.

thus allowing a better precursor/cell interaction. These difference could be due that bacteria be genetically favoured to carried out this biotransformation in aerobic condition.

The spectroscopic data for compound I from experiments with *E. coli* are identical to those reported by Atkinson et al. [24] and this compound was identified as D-DIBOA. The transformation of the precursor can be explained in terms of the presence of nitroreductase enzymes, which are involved in the reduction of the nitro group of the precursor.

The biotransformation could be similar to that involved in the reduction of TNT to the corresponding amines [17] as both compounds contain an aromatic ring and a nitro group at C'5. In addition, *E. coli* is able to reduce these nitroaromatic compounds, most of which are toxic and mutagenic, to the corresponding amines through three types of nitroreductase [25]: two oxygen-insensitive (Type I) and an oxygen-sensitive (Type II) [26] type. In our case, it is possible that type I is involved as the biotransformation occurs equally well in both oxygenic and anoxygenic conditions.

As far as biotransformation yields are concerned, the biological process is still inefficient. These results highlight several variables that could be changed in future studies to achieve better operating performance: e.g. the culture medium composition, the concentration of precursor, the effect of iron concentration, the agitation rate and the purification method.

In conclusion, these results confirm that the strain *E. coli* used in this study is capable of biotransforming the precursor to D-DIBOA, although the yield of this biotransformation is still low. The results also suggest that the enzymes of *E. coli* nitroreductases, as described in the literature, may be present in the strains used and may participate in the reduction of the nitro group of the precursor. The objectives for future work are the identification of the enzymes responsible for the biotransformation using molecular biology techniques and the optimization of operational variables in order to increase the production yields.

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