Biotransformation of Pharmaceuticals by Comamonas and Aeromonas Species

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Research Article

Keywords: Antibiotic resistance, Biotransformation, Erythromycin, Sulfamethoxazole-trimethoprim, Comamonas jiangduensis, Aeromonas hydrophila, Aeromonas caviae

Posted Date: January 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-144884/v1

Abstract

Background: Contamination of natural niches with pharmaceutical residues has emerged out as a serious concern. Disposal of untreated effluents from the pharmaceutical, hospital, and domestic settings has been identified as a significant source of such a massive spread of antibiotics. The unnecessary persistence of pharmaceutical residues including antibiotics has been related to the increased risk of resistance selection among pathogenic and non-pathogenic microorganisms. To date, several methods have been devised to eliminate such pollutants from wastewater, but their implication on larger scales is not feasible due to complexities and high costs of the processes, especially in developing and underdeveloped countries. This study aimed to isolate and characterize bacterial strains from domestic and pharmaceutical effluents having biotransformation potential towards most persistent antibiotics.

Results: Antibiotic resistance screening and MIC determination experiments indicated highest resistivity of three bacterial isolates against two antibiotics Erythromycin and Sulfamethoxazole-trimethoprim, evincing extensive usage of these antibiotics in our healthcare settings. These isolates were identified as *Comamonas jiangduensis, Aeromonas caviae and Aeromonas hydrophila* by 16S rDNA sequencing. Growth conditions including incubation temperature, initial pH and inoculum size were optimized for these strains. Successful biotransformation of Erythromycin and Sulfamethoxazole-trimethoprim was achieved within 92 h under optimum growth conditions.

Conclusions: *Aeromonas* and *Comononas* species were found to be potent degraders of antibiotics tested, presenting these strains as potential candidates to be utilized in the remediation processes.

Background

The discovery of antibiotics in 19th century played a crucial role in the treatment of life threatening infections and drastically altering the mortality and morbidity rates associated with them (1). Since then, these biologically active compounds have become indispensable in the maintenance of human health and improvement of quality of life. Besides human medicine, antibiotics have extensive application in aquaculture research and veterinary medicine to treat infections and promote growth in animals (2). The global consumption of antibiotics is reported to be increased by 65% from 21.1 to 34.8 billion DDD (defined daily doses) from 2000 to 2015 (3). Such widespread usage of antibiotics has given rise to concerns related to their persistence in the environment for longer periods of time (4). This is attributed to their partial metabolism in humans and animal body which results in their discharge in active form (5). The unnecessary persistence of antibiotics results in the development of drug resistance in human, animals and pathogenic microorganism, rendering the drugs ineffective against the diseases (6). Apart from pathogenic resistivity, antibiotics also have adverse effects on structure and functions of ecological system. Biological diversity plays key role in the maintenance of ecological functions and biogeochemical cycles, which are generally mediated by a consortium of microorganisms. Antibiotics reduces biodiversity by eliminating or inhibiting non-target microbial species, ultimately affecting normal biological processes such as nitrogen fixation, biomass production and functional stability (7).

Domestic, pharmaceutical and hospital effluents are the primary sources of antibiotic disposal and are considered as the hot spots for their widespread release in the environment (8). If these effluents are not properly treated in sewage treatment plants for the degradation and elimination of such active compounds, they ultimately find their way into the natural environment such as soil, surface water and ground water (9). Several studies have reported the presence of a wide range of antibiotics in natural water resources as well as wastewater treatment plants (10, 11,12,13).

Various Non-biotic and chemical methods have been devised and employed to date to eliminate organic pollutants including advanced oxidative and adsorptive processes (14, 15,16). Complexity, high costs and difficulty of chemical sludge disposal of such processes render them inefficient for mass scale application (17). Over the years, biotransformation has emerged out as a safe and cost-effective tool for acclimatizing the environment and has been employed to transform a wide range of organic pollutants including metals, pharmaceuticals and hydrocarbons. It is majorly because of the observed catabolic

diversity, growth rate and the property of horizontal gene transfer of microbes, representing them as a suitable candidate for this process (18).

The majority of biotransformation studies have utilized mixed cultures isolated from sewage sludge or soil sources. Biodegradation of sulfonamides has been achieved by bacterial strains isolated from sewage sludge including *Achromobacter denitrificans* PR, *Pseudomonas* and *Arthrobacter* species (19, 20, 21). Liao and his co-workers investigated the ability of mixed culture consisting of bacterial strains belonging to classes Bacteroidia, Gammaproteobacteria and Betaproteobacteria to degrade ciprofloxacin (22). A consortia named AMQD4 was found for the removal of gentamicin from waste produced during its production (23). However, limited number of studies have reported the biotransformation of individual antibiotics by pure cultures other than fungal isolates (24).

The current study is based on the isolation and molecular identification of antibiotic-resistant bacterial strains from domestic and pharmaceutical effluents, having the potential to carry out significant degradation of most persistent antibiotics in the environment. Optimization of parameters such as pH, temperature, inoculum size and growth time was carried out to achieve maximum degradation of antibiotics. Furthermore, biotransformation efficiency of these isolates was evaluated to develop an efficient and cost-effective strategy for large scale remediation of polluted environments.

Results

Isolation and antibiotic resistance screening of bacterial strains

A total of 12 strains were isolated, of which five were obtained from pharmaceutical and seven from domestic effluents. All strains were grown on general-purpose, selective, and differential media. Isolated strains were tested for their resistance against five antibiotics, including ERY, STRP, CIP, SXT, and MET. Of 12 strains, 9 showed complete resistance to SXT as no inhibition zone was observed around the antibiotic disc. For ERY, 4 isolates showed absolutely no inhibition zone, whereas 3 isolates showed zones within the selective range for resistance. Only one strain showed resistance to CIP, and the same was the case with STRP. None of the isolates showed resistance against MET. Six isolates PC, PD, PE, D1A, D2D, and D2F, showed the highest resistance against SXT and ERY and were selected for further experiments. These results indicated the presence of multiple drug resistance patterns among these isolates except D2D, which was resistant to SXT only. The antibiotic resistance profile and percentages of all 12 isolates are given in Figure 1A and 1B.

MIC of antibiotics for resistant strains

The MIC of ERY and SXT for six selected isolates is summarized in Table 2. For ERY, three strains, PC, D2F, and D1A, showed the highest resistance with the MIC of 512 µg/ml. Same strains, D1A and D2F, were observed to be highly resistant to SXT as well, the highest MIC observed for both strains was 1024 µg/ml.

Identification of isolated strains:

Molecular characterization of antibiotic resistant strains:

Amplicon size for all three bacterial strains was ~200 bps (Figure 2). According to BLAST analysis, isolate PC, D1A, and D2F displayed the highest sequence similarity to *Comamonas jiangduensis*, *Aeromonas hydrophila,* and *Aeromonas caviae*, respectively. The accession no. for strain PC is MN587995, D1A is MN587996 and D2F is MT560322. A phylogenetic tree was constructed for all three isolates to determine their evolutionary relationship with closely related species (Figure 3A and 3B).

Optimization of culture conditions:

Optimization of culture conditions, including temperature, pH, and inoculum size, was done for the three highly resistant strains PC, D1A, and D2F, to achieve maximum biotransformation of antibiotics. Optimization of incubation temperature was carried out by incubating NB tubes containing antibiotics (MIC) and culture from selected isolates at different incubation temperatures, including 30, 35, 37, 40, and 45°C. A similar temperature requirement was observed for the maximum growth (OD600) of all three isolates irrespective of the presence of either ERY or SXT. For ERY, the temperature at which all three strains, PC, D1A, and D2F, showed maximum growth was found to be 35°C (Figure 4A). Maximum growth in the presence of SXT was also observed at 35°C for both D1A and D2F (Figure 4B). However, the growth of all three isolates decreased dramatically with further increase in temperature.

Maximum growth of strains PC and D1A was obtained at pH 7.5 whereas, strain D2F gave highest OD at pH 7 in the presence of ERY. A significant decrease in growth was observed with the increase in pH from 7.5 to 9.5 (Figure 5A). The optimum pH for the growth of strains D1A and D2F in the presence of SXT was found to be 9, where OD600 drastically increased from pH 5.5 to 9 and decreased afterward (Figure 5B).

For all three strains, varied inoculum size requirements were observed, suggesting its significant role in growth kinetics of bacteria. In the presence of ERY, strains PC, D1A and D2F showed maximum growth at 0.66, 1.66, and 0.066%, respectively (Figure 6A). Surprisingly, the two strains D1A and D2F, which were resistant to ERY as well, also showed different inoculum size requirement of 1% for maximum growth in the presence of SXT in contrast to ERY (Figure 6B).

The growth curve of antibiotic-resistant strains was studied to determine their viability in culture medium over the course of time and the events (lag, log, stationary, and decline phase) taking place in the bacterial population. For all three isolates, the lag phase was observed for an hour, which shifted towards the log phase with an exponential increase in cell densities till 72 h after which stationary phase was achieved as no significant increase in the OD was observed afterward. These results indicated that the time required to reach the stationary phase is not affected by the presence of two different antibiotics, even for the common strains (Figure 7A and 7B).

Determination of biotransformation of antibiotics

Microbiological assay for the evaluation of antibiotic potency

Reduction in the potency of antibiotics treated with bacterial strains was observed by reduction in inhibition zone over time. These zones were compared to the standards of known antibiotic concentrations (Table 3) to assess the extent of reduction in test antibiotic's antimicrobial activity. For ERY, Isolate PC showed a significant reduction in the initial concentration (512 µl) within 3h of incubation. This concentration reduces to another half after 24 h, and a much smaller inhibition zone was observed at 96 h (Figure 8). Almost similar results were obtained with the strain D2F for ERY (Figure 8). More than a 2-fold reduction in ERY concentration was observed after 3h of incubation with the strain D1A, which drastically reduced in further 24 h of incubation. A very slight zone was observed around the test sample after 96 h of incubation (Figure 8). For SXT, strain D2F showed an almost 4-fold reduction in the initial concentration (1024 µl) within 3h of incubation, which decreased further by the factor of 2 in 24 h and ultimately a very slight to a negligible zone of inhibition was observed at 96 h (Figure 9). Strain D1A showed similar pattern of reduction of SXT as for ERY in the three studied time intervals with almost complete reduction in zone of inhibition within 96 h (Figure 9).

In summary, obvious biotransformation of ERY and SXT was achieved by PC, D1A, and D2F.Out of three strains, D1A was found to be the most potential candidate for this purpose as no considerable inhibition of susceptible strain was observed around the test sample of maximum growth time (96 h).

Discussion

Pharmaceuticals have been present as micro-contaminants in our environment for the past few decades (25). Their persistence in aquatic environments had become a significant threat to human and veterinary health as well as ecological sustainability (26). Despite recent advances in the development of wastewater treatment processes, the presence of antibiotics as a potential risk factor for conferring resistivity has not yet been overcome. Extensive research has been done to isolate and identify antibiotic-resistance bacterial strains from different environmental sources (27, 28, 29). However, little is known about the antibiotic degradation potential of these bacterial strains for the environment's bioremediation, resulting in significant suppression of antibiotic resistance proliferation in susceptible microbial communities (30).

This study evaluated the biotransformation potential of antibiotic-resistant bacterial strains isolated from pharmaceutical and domestic effluents. Results from initial antibiotic resistance screening and profiling experiments indicated a majority of gram-negative isolates to be highly resistant to tested antibiotics showing, the burden of resistance among this group. Recent studies have evaluated the burden of infections caused by multiple drug-resistant (MDR) gram-negative bacteria, and it has been observed that the occurrence of these infections has even surpassed the ones caused by gram-positive MDR pathogens including, *MRSA* (31). Of all five antibiotics tested, the highest resistance was observed for two antibiotics ERY and SXT. The emergence of resistance against these antibiotics is attributable to their extensive use in the treatment of routine clinical infections. Both antibiotics belong to the two different classes of antibiotics and have a different mechanism of action. SXT is effective against both gram-positive and negative bacteria and imparts its bactericidal effect by disrupting the folate pathway (32) whereas, ERY acts by inhibiting protein synthesis and is most effective against gram-positive and also against very few gram-negative bacteria (33). As all three studied isolates are gram-negative, resistance against SXT could be due to the transfer of resistant genes between gram-positive and negative bacteria, as described in some studies (34).

MIC experiments selected for three isolates PC, D1A and D2F having highest resistance against ERY and isolates D1A and D2F having highest resistance against SXT. Molecular characterization of three isolates PC, D1A, and D2F by 16S rDNA sequencing identified them as *Comamonas jiangduensis, Aeromonashydrophila,* and *Aeromonas caviae,* respectively. *Comamonas jiangduensis* belongs to the class *Comamonadaceae*. There are very few studies regarding the pattern of antibiotic resistance in *Comamonas* species. This strain was found to be highly resistant to ERY with the MIC of 512 µg/ml in the current study. Resistance to β-lactams has been reported in the *Acidovorans* strains of *Comamonas* (35, 36). *Comamonas* species have also been associated with the bioremediation of heavy metals such as Cadmium and other organic pollutants (37, 38, 39). In Particular, *C. jiangduensis* has been identified as the most active strain of a carbofuran degrading consortium (40). Moreover, *Comamonas jiangduensis* has also been reported to produce biosurfactants, elucidating their potential to serve as a candidate for bioremediation of organic compounds (41).

The other two strains, *Aeromonashydrophila* and *Aeromonas caviae,* belong to the family *Aeromonadaceae*. They are facultative anaerobes and are usually present in aquatic environments, including fresh and brackish waters (42). Incidence of resistance has been reported against many antibiotics classes, including penicillin, due to an enzyme beta-lactamase (43). *A. hydrophila*, isolated from the aquatic environment, was resistant against ten antibiotics, including erythromycin, streptomycin, trimethoprim, and penicillin, suggesting its multidrug-resistant nature (44). Very high values of MIC were observed in this study against ERY and SXT (512 µg/ml and 1024 µg/ml respectively) for *A. hydrophila* indicating the presence of a powerful resistance mechanism. *A. hydrophila* has been employed for the bioremediation of wastewater contaminated with dyes frequently used in textile industries. In a study, up to 96% detoxification of triarylmethane dyes was achieved by *A. hydrophila* within 24 h. The operational conditions most suitable for this degradation were 35°C and a pH range of 7-8. Less toxic metabolites were produced after the degradation of these dyes (45). Another toxic organic compound, Reactive black 5 present in wastewater from the textile industry, was degraded and decolorized by the action of *A. hydrophila* under an optimum temperature of 35°C and pH 7. About 76% of degradation was achieved within 24 h (46). Optimum growth conditions required in these studies to eliminate dyes can be compared to the one obtained in our study, indicating more or less similar requirements of *A. hydrophila* to carry out biodegradation of organic pollutants. Few reports

have indicated the presence of *Aeromonas caviae* strains as part of a consortium for the biodegradation of organic pollutants such as linear alkylbenzene sulfonate and hydrocarbon from water bodies (47, 48).

Growth parameters such as temperature and pH were optimized to harvest maximum elimination of antibiotics by microbes. Temperature plays a significant role in the degradation of pollutants by bacterial strains as it directly effects the activity of enzymes involved in the degradation of such compounds as a mechanism of resistance (49) It is also essential for the growth and maintenance of bacterial cells' cell structure (23). Several studies have reported the temperature range of 30-35°C as the most suitable range for biodegradation of organic pollutants (23, 50, 51). Similar results were observed in the biotransformation of ERY and SXT by strains PC, D2F, and D1A, where all three of them showed maximum growth at 35°C. In addition to temperature, the medium's initial pH also plays a significant role in microbes' survival and metabolic activities (52). In this study, the three isolates PC, D1A and D2F showed maximum growth and degradation of ERY at a pH range of 7 to 7.5. In contrast, strains D1A and D2F showed maximum growth at pH 9 in presence of SXT. Initial alkaline pH requirement of 9 was also observed for degradation of tetracycline at 30°C (53). Almost similar pH requirements were observed for the degradation of a pesticide, methyl parathion, by the action of *P. aeruginosa, P. diminuta and P. putida*, where maximum degradation was obtained at pH 8.5 (54). Such a difference in the pH requirement of the same strains in the presence of different antibiotics in this study is strange but could be speculated to be due to the difference in both antibiotics' physicochemical properties or the active enzyme for degradation of SXT might require an alkaline pH to impart its catalytic activity.

Inoculum size is also a pivotal factor for the growth of microorganisms and can significantly influence their degradation efficiencies towards contaminants (55). In our study, inoculum size was significantly associated with bacteria's growth and the degradation of antibiotics as the inoculum dosage requirement differed with each strain and antibiotic. These results are in contrast to the studies where maximum degradation was achieved with an increase in inoculum size (51) or which concluded no significance of inoculum size on the degradation efficiencies (23).

Growth curve analysis is also of great importance for bioremediation and commercial implementation of microbes. It helps to identify a microbe's ability to metabolize a particular substrate as an energy source and its potential to survive in the presence of toxic compounds (56). The three bacterial strains under study showed an increase in cell density till 72 h in the presence of SXT and ERY, after which the stationary phase was achieved. Several factors, including nutrient imbalance and depletion and accumulation of toxic compounds, are considered to be the possible reasons that bring bacterial growth to a halt over a specific time (57).

In this study, biotransformation of antibiotics was evaluated by reduction in potency with time, which served as an indicator of their transformation to non-toxic compounds. All three strains showed a remarkable transformation of both antibiotics within four days of incubation. Of all three, strain *A. hydrophila* (D1A) was found to be the most potent degrader of both antibiotics. Similar approach has been utilized to evaluate the reduction in antimicrobial potency of tetracycline by a gram-negative strain *Stenotrophomonas maltophilia* DT1. Maximum biotransformation of tetracycline was observed within first four days of incubation which corresponds to our study. (53). Moreover, a microbiological assay was a straightforward, robust, and inexpensive method compared to the routinely used approaches such as spectrophotometric detection and High pressure liquid chromatography (HPLC) and the results obtained are reproducible (58).

Conclusion

To the best of our knowledge, this is the first study that examined the potential of three pure bacterial strains, *Comamonas jiangduensis, Aeromonas caviae, and Aeromonas hydrophila*, isolated from pharmaceutical and domestic effluents for the biotransformation of two antibiotics, ERY and SXT. The optimum temperature for the maximum growth of all three strain was 35 ºC, whereas pH and inoculum size requirement varied for all isolates in the presence of both antibiotics. Evaluation of biotransformation by microbiological assay indicated the successful transformation of both the antibiotics within 92h. Of all three isolates, *Aeromonas hydrophila* was found to be the most potent degrader of both antibiotics. Furthermore, a

microbiological assay was the most robust and inexpensive method to determine the reduction in potency of antimicrobials compared to HPLC and other spectrophotometric approaches. Isolated bacterial strain can be used in a consortium to target these antibiotics contaminating environmental niches after further extensive studies. Moreover, studies can be conducted to reveal the genetic basis of the transformation mechanism. Determining the genes and metabolic pathways involved in the transformation of antibiotics will help develop genetically engineered microbes with additional qualities and more controlled regulation.

Methods

Chemicals and reagents:

Erythromycin (ERY) and Sulfamethoxazole-trimethoprim (SXT) were purchased from a local pharmacy with brand name Erythrocin (500mg) and Septran (80/400mg) respectively. Media including Nutrient broth (NB), Nutrient agar (NA), Mannitol Salt agar (MSA), MacConkey agar (MCA), Eosin Methylene Blue (EMB) agar and Muller-Hinton agar (MHA) were purchased from Sigma Aldrich. All other chemicals and reagents used were of analytical grade purchased from commercial sources.

Sample collection and isolation of bacterial strains:

Pharmaceutical effluent and domestic wastewater samples from two different sewers were collected to screen and isolate antibiotic-resistant bacteria as they are considered major reservoirs of partially metabolized antibiotics, giving rise to resistant mutants. Samples from one pharmaceutical and two domestic sewage sites were given names as P1, D1 and D2. To isolate bacterial strains from effluent samples, general purpose as well as selective and differential media including, NA, MSA, MCA and EMB were used. 10µl of each sample was inoculated in petri plates having aforementioned media and incubated at 37°C for 24 h. Post incubation, 5 colonies were selected randomly from each plate, having distinct colonial morphology from each other. These colonies were inoculated on separate petri plates, containing the same growth medium from which they were picked to obtain a pure culture.

Screening for antibiotic-resistant strains

Isolates were screened for their resistivity to 5 different antibiotics, namely Ciprofloxacin (CIP) (5µg), Erythromycin (15µg), Streptomycin (STRP) (10µg), Sulfamethoxazole-trimethoprim (25µg) and Metronidazole (MTZ) (5µg) by classical Kirby-Bauer disc diffusion test. These antibiotics were selected as the representatives of five major classes including floroquinolones, macrolide, aminoglycosides, co-trimoxazole/sulfonamide and nitroimidazole. Zones of inhibition for all five antibiotics were measured and isolates were categorized as susceptible, intermediate and resistant based on Clinical and Laboratory Standards Institute (CSLI) (59). Zone diameter breakpoints for the selection of antibiotic-resistant isolates are given in Table 1.

Determination of minimum inhibitory concentration (MIC)

The MIC of selected antibiotics for resistant isolates was determined by the Agar dilution method proposed by Andrews (60). The inoculum was prepared by inoculating NB with 2 to 3 colonies of the strains and kept for incubation in a shaking incubator at 150 rpm and 37°C for 24 h. Turbidity of bacterial suspensions was adjusted to 0.5 McFarland's standard. Two-fold serial dilutions of antibiotics were prepared from the stock solution and appropriate volume of these antibiotics was incorporated in separate flasks containing NA medium. Antibiotic-containing media was poured on plates labelled with respective antibiotic concentration. 2 µl of bacterial suspension was inoculated to the plates and incubated at 37°C for 24 h.

DNA extraction

Selected antibiotic-resistant strains were grown in 15 ml NB for 48 h at 37°C in a shaking incubator to obtain maximum growth. After incubation, bacterial cells were palleted down by centrifugation at x13000 g for 10 min. The cell pallet was washed twice with sterile Phosphate buffered saline (PBS) by centrifugation at x13000 g for 10 min each time. Washed cells were subjected to lysis by adding 650 µl of lysis buffer (1% SDS, Tri-HCl 100mM pH-8, Na- EDTA 50mM pH-8 and RNase A 10mg/ml) followed by vigorous mixing for 5 min. The mixture was centrifuged at x13000 g for 10 min with subsequent transfer of 500 µl of supernatant to the fresh tube containing 100 µl of Potassium-acetate buffer (3M). After centrifugation (3 min x13000 g), supernatant (500 µl) was transferred to another tube containing isopropanol and inverted several times to make the bacterial DNA insoluble. The tube's contents were then centrifuged (2 min at x13000 g) to obtain a DNA pallet. The supernatant was discarded, and 750µl of 70% ethanol was added to wash the DNA pellet and centrifuged (30 sec at x13000 g). DNA pallet was air-dried, re-suspended in 50 µl of sterile distilled water, and stored at -20°C for further experiments. Concentration and purity of extracted DNA samples were assessed using Nanodrop (Thermo Fisher Scientific).

PCR amplification and Sequencing

Amplification of highly conserved, variable (V3) region of 16S rRNA bacterial genes was carried out using a set of 16S rDNA general primer (10 µM) 5'-CCTACGGGAGGCAGCAG-3' and 5'- ATTACCGCGGCTGCTGG-3' (61). For each amplification reaction, DreamTaq master mix (Thermo Fisher Scientific, USA) was used. Reaction mixture (25 µl) was prepared by adding 12.5 µl master mix, 9.5 µl RNase free water, 1 µl of each forward and reverse primers and 1µl of DNA sample. Gene was amplified by using following PCR cycles; initial denaturation (95°C for 5 min), 30 cycles of denaturation at 95°C for 30sec, annealing at 61°C for 30sec and extension at 72°C for 30sec and final extension for 5min at 72°C. The amplified product was visualized by Agarose gel electrophoresis (1.5%) at 118 Amp for 30 min. DNA bands were analyzed using Gel Doc (BIO-RAD, USA) and compared to the 1kb reference ladder. Amplified gene was extracted and purified from agarose gel by a kit-based method (Thermo Fisher Scientific, USA) and sent to Centralized science laboratories, the University of Karachi, for sequence analysis using an ABI Prism® 3130 Genetic analyzers (Applied Biosystems, Hitachi Japan). Obtained sequences were viewed using BioEdit Sequence Alignment Software (Ibis Biosciences, USA), subjected to BLAST analysis for pairwise comparison using the NCBI database, and submitted to GeneBank.

Studies on growth conditions for biotransformation

Inoculum preparation

NB was inoculated with 2-3 colonies grown on NA and kept at 37°C for 24 h in a shaking incubator. Optical density ($OD600_{)}$ was recorded for each bacterial strain to obtain the number of bacterial cells present per ml (1.5×10^8 cells/ml).

Optimization of culture conditions

Optimization of conditions most suitable for biotransformation of antibiotics was carried out with single-factor tests. The parameters optimized were initial pH (5.5 to 10.0), incubation temperature (20 to 45°C), and inoculum size (0.066 to 2.35%). Growth curve analysis of all isolates was also performed (0 to 96 h). The inoculum was prepared by matching with a 0.5 McFarland standard. NB tubes (15 ml) were supplemented with the MIC of selected antibiotics separately, and each tube was inoculated with 100µl of inoculum. Incubation was done at an optimized temperature for 24 h. The growth of each resistant strain was evaluated by spectrophotometric analysis (OD600). The condition at which highest growth of antibiotic resistant strain was achieved was used for further optimization of parameters.

Determination of biotransformation of antibiotics

Biotransformation of antibiotics by resistant isolates under optimum culture conditions with time was evaluated by a microbiological assay (62). For this purpose, fractions of culture medium (2ml) were collected during growth curve analysis at different time intervals (3, 24, and 96 h). These fractions served as test samples (unknown concentration) as they contain

antibiotics that have been acted upon by the bacterial strains having specific mechanisms to resist their bactericidal effect. The test samples were first exposed to chloroform for 24 hours to kill any viable bacterial cells. Standard samples consisting of two-fold dilutions of antibiotics (known concentration) were prepared from the stock solution to serve as a reference against test samples. To assess the reduction in antimicrobial potency of the antibiotics (ERY and SXT) over time, NA plates were streaked with the bacterial strain susceptible to both the antibiotics. Sterile absorbent paper discs were prepared and impregnated in three standard samples and 3 test samples obtained from individual strains. These discs were then placed on the plates (prepared for each isolate and antibiotic separately) and incubated at 37°C for 24 h. This experiment's results were assessed by the observation of inhibition zones around the discs of both standard and test samples.

Statistical analysis

Results obtained from screening, optimization and transformation evaluation experiments were analyzed statistically using MS Excel 2013. Means and standard deviations were calculated as each test was performed in duplication to minimize error chances.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: The data used during the current study is available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests

Funding: Financial support provided by the Shaheed Zulfikar Ali Bhutto Institute of Science and Technology (SZABIST), Karachi, Pakistan.

Authors' contributions: 1- Atika Sajid: Performed the experiments, wrote the paper.

2- Saira Yahya: Research idea, Supervised the project, in-charge of overall direction and planning, writing and revision of the manuscript

Acknowledgements: Not applicable

Authors' information (optional): Not applicable

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Tables

Table 1: Zone diameter breakpoints for selecting bacterial strains Resistant, Intermediate, or Susceptible to antibiotics.

Antibiotic	Resistant < or = (mm)	Intermediate (mm)	Susceptible > or = (mm)
Ciprofloxacin	15	16-20	21
Erythromycin	13	14-22	23
Streptomycin	14	15-20	21
Sulfamethoxazole-trimethoprim	10	11-15	16
Metronidazole	N/A		

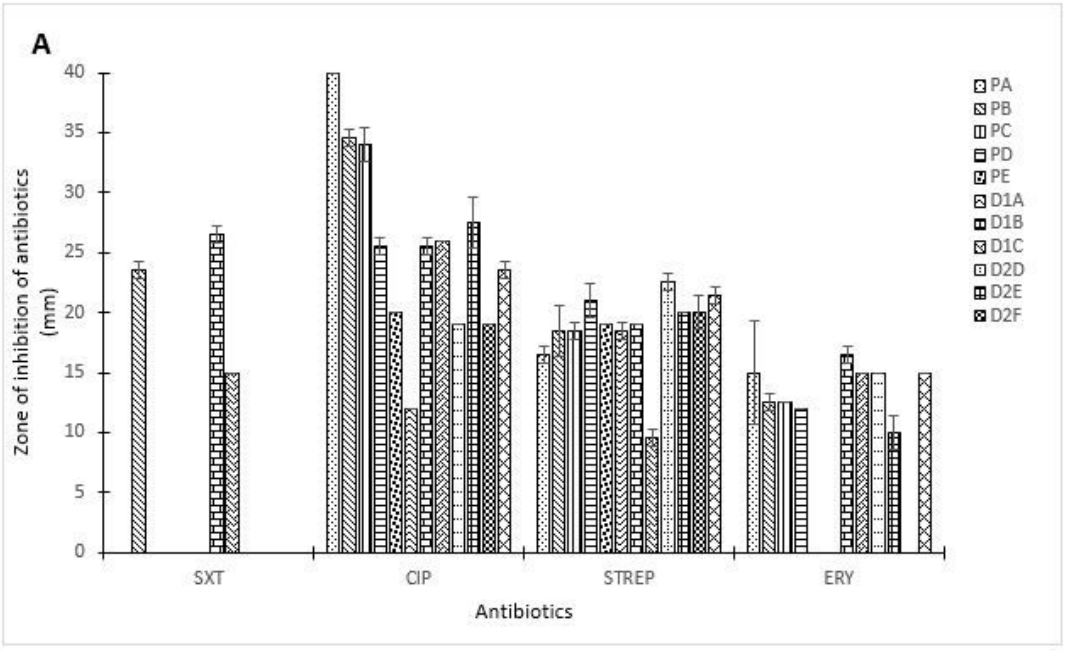
Table 2: MIC of Erythromycin and Sulfamethoxazole-trimethoprim of isolated strains.

Isolate code	Dilution of Erythromycin (µg/ml)								Dilution of Sulfamethoxazole-trimethoprim (µg/ml)								
	8	16	32	64	128	256	512	1024	8	16	32	64	128	256	512	1024	2048
PC	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-
PD	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
PE	+	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-
D1A	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
D2D	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
D2F	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-

Table 3: Mean diameters of the inhibition zones obtained for ERY and SXT standard solutions

Antibiotic	Standard Sample	Concentration (µg/ml)	Zone diameter (mm)
Erythromycin	S1	512	32.5 ± 0.707
	S2	256	26 ± 0
	S3	128	20.5 ± 0.707
Sulfamethoxazole-trimethoprim	S1	512	40.5 ± 0.707
	S2	256	31.5 ± 0.707
	S3	128	22 ± 0.707

Figures



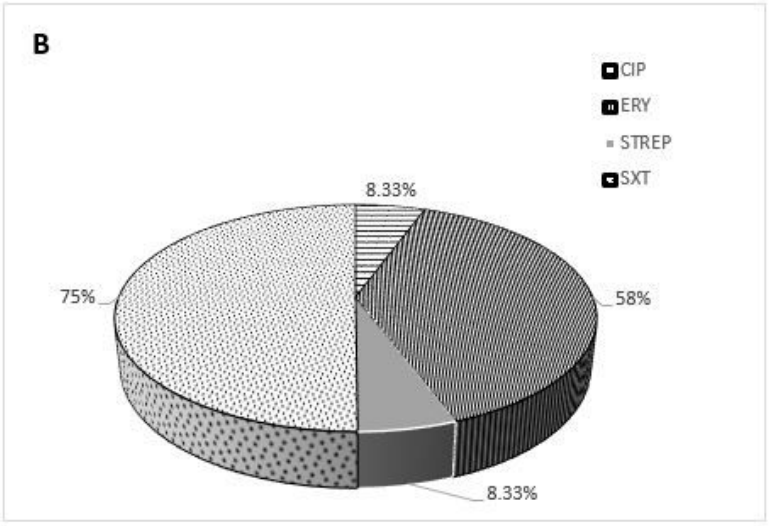


Figure 1

(A) Bar graph showing mean diameter of zones of inhibitions for antibiotics against bacterial isolates. (B) Pie diagram representing distribution of resistance against antibiotics among bacterial isolates.

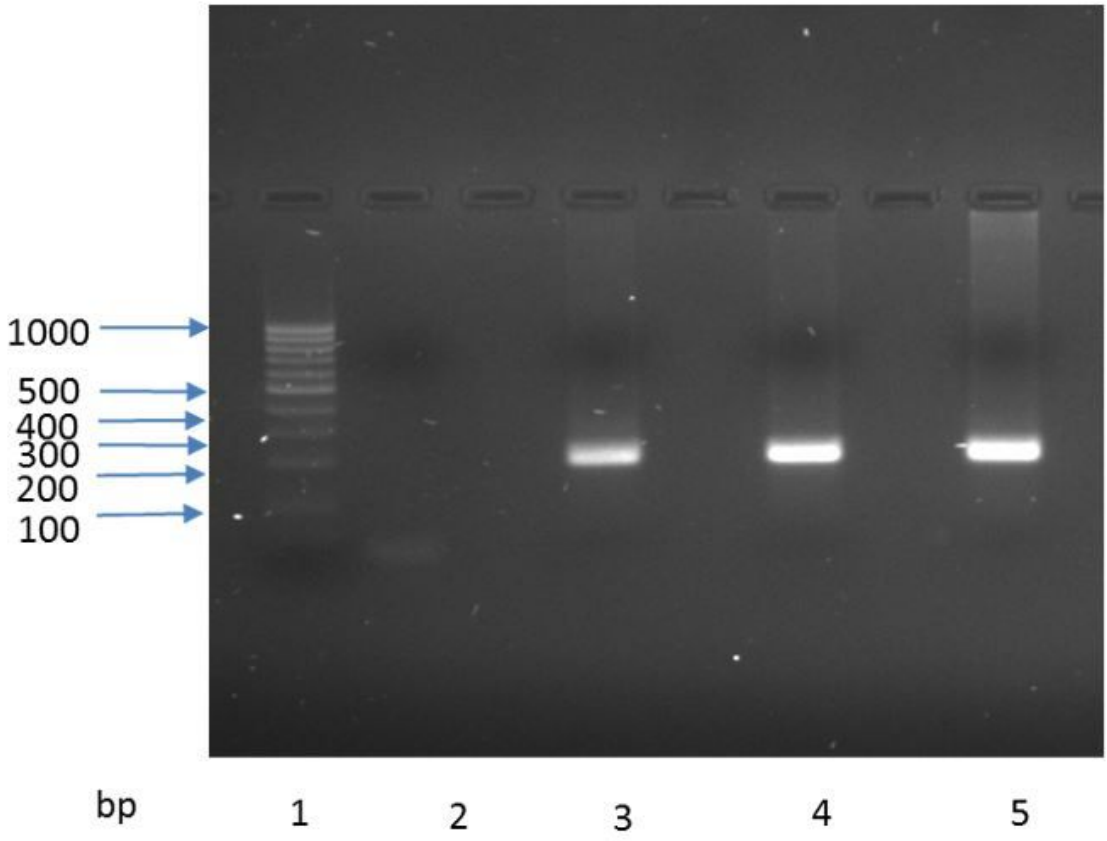


Figure 2

Gel electrophoresis of PCR amplicons obtained from DNA isolated from three antibiotic resistant strains using 16S rDNA general primers. Lane 1: reference ladder (1 kb), Lane 2: Negative control, Lane 3-5: PCR products of isolates PC, D1A and D2F respectively.

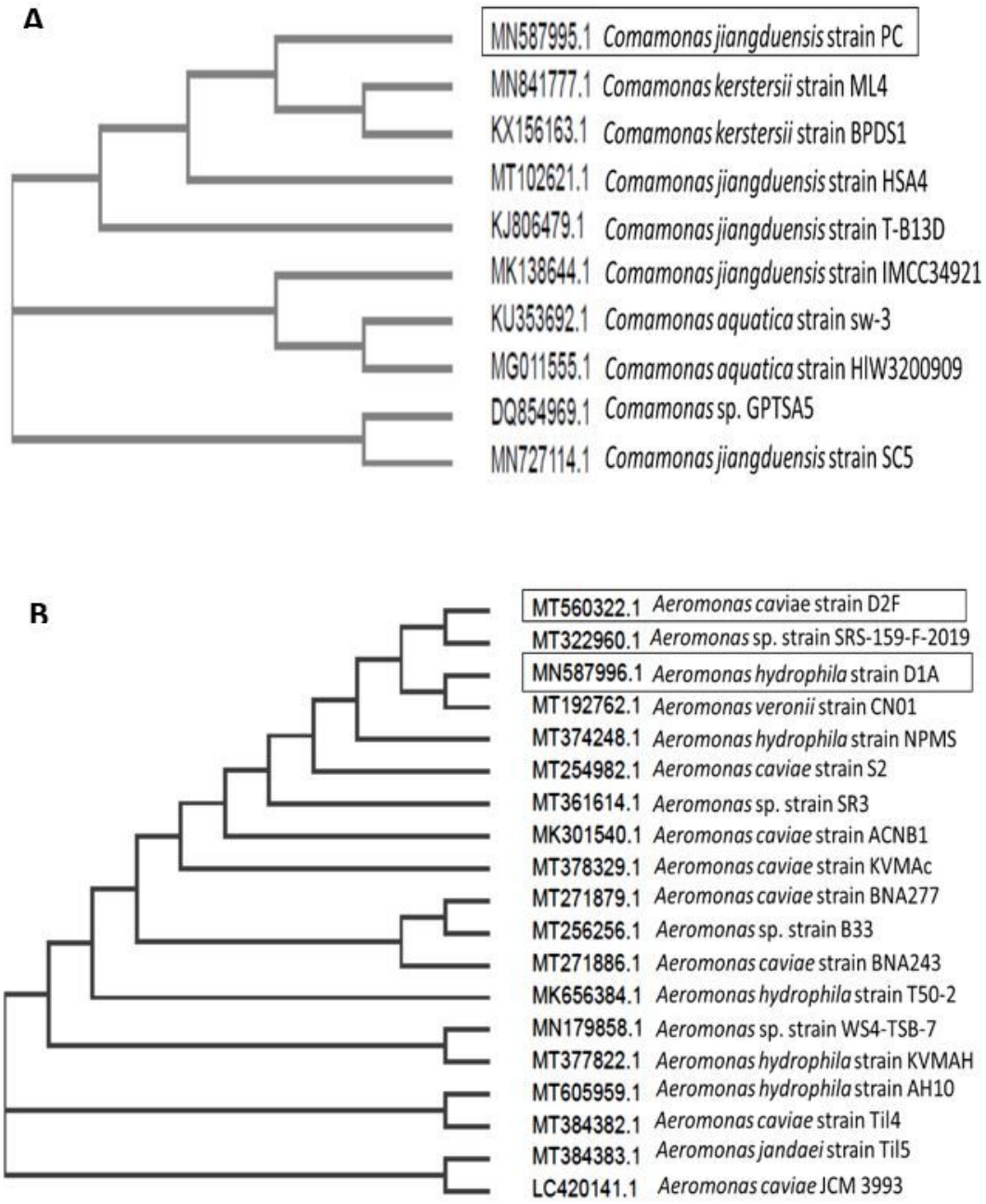
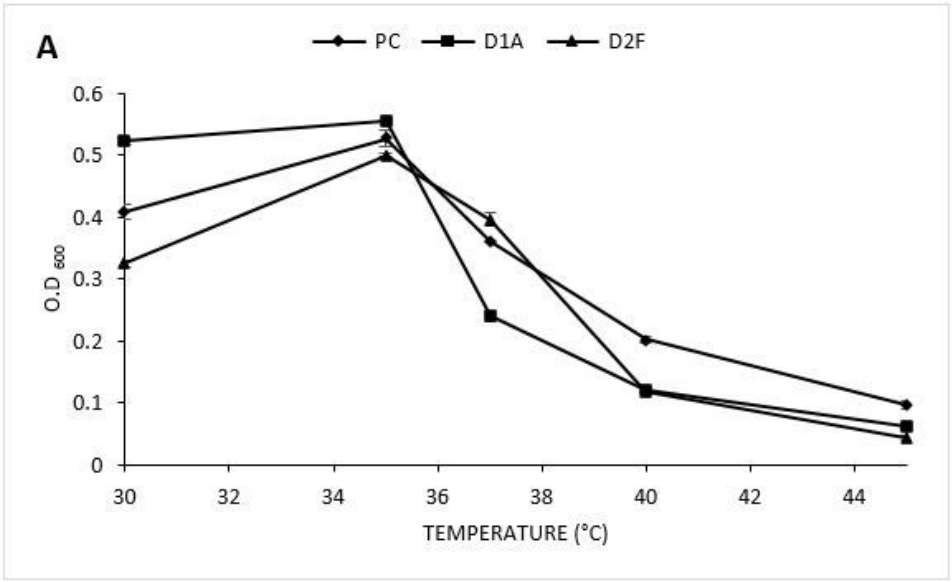


Figure 3

Phylogenetic tree for Bacterial strain PC (A) and D1A and D2F (B) based on 16S rRNA gene sequence.



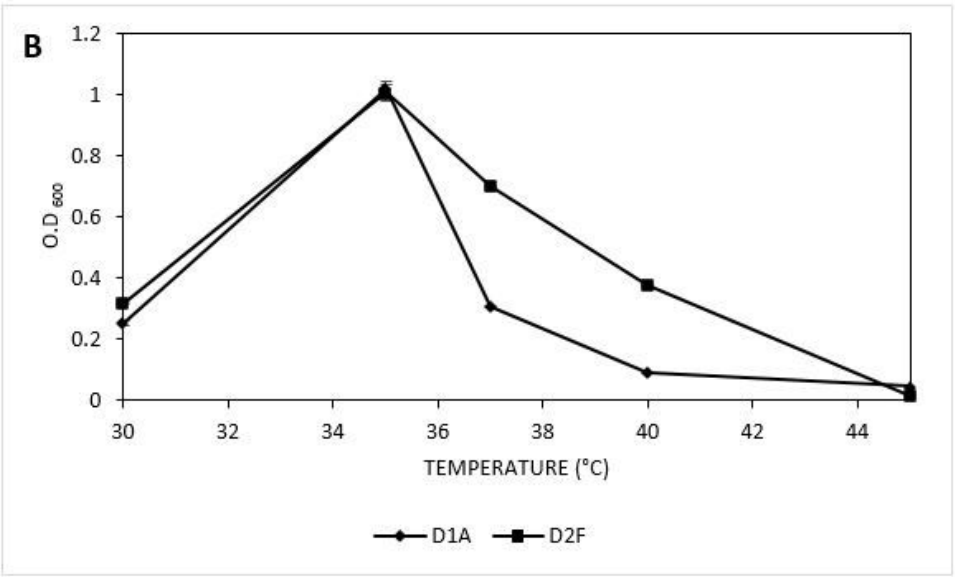
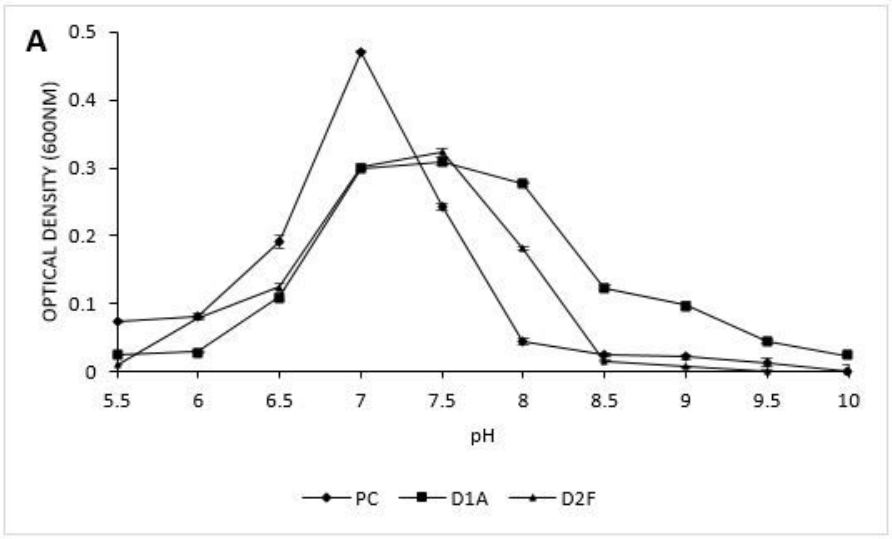


Figure 4

Effect of incubation temperature on growth of bacterial strains (A) PC, D1A and D2E in the presence of ERY and (B) D1A and D2F in the presence of SXT.



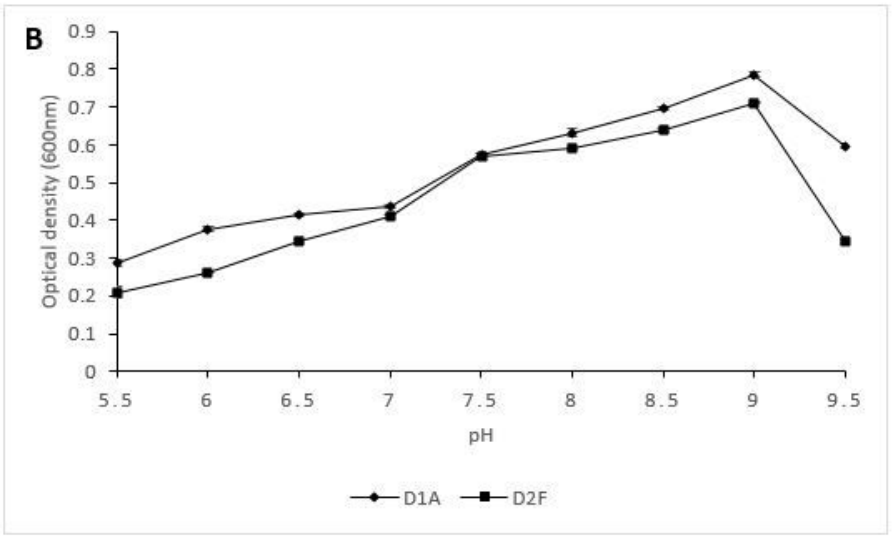
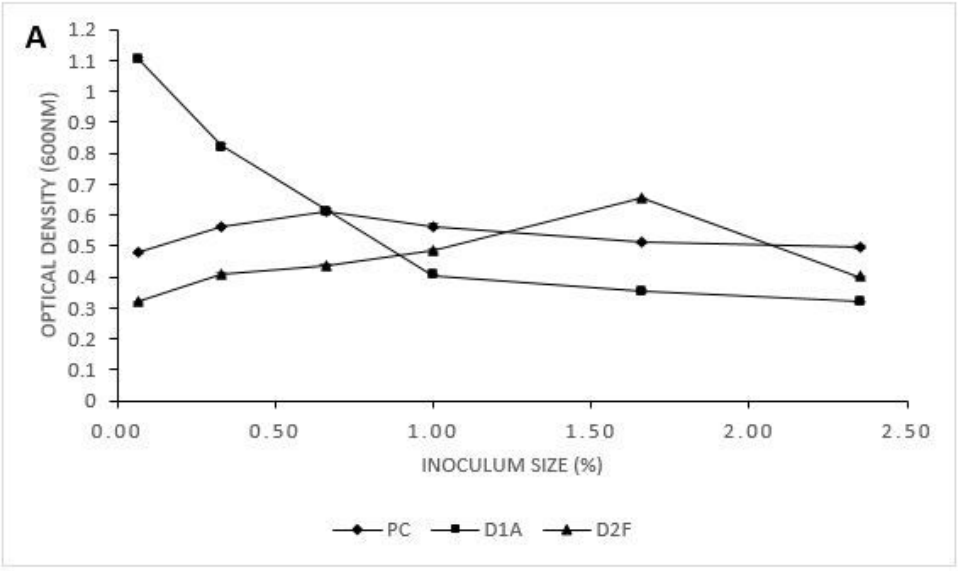


Figure 5

Effect of pH on growth of bacterial strains (A) PC, D1A and D2E in the presence of ERY and (B) D1A and D2F in the presence of SXT.



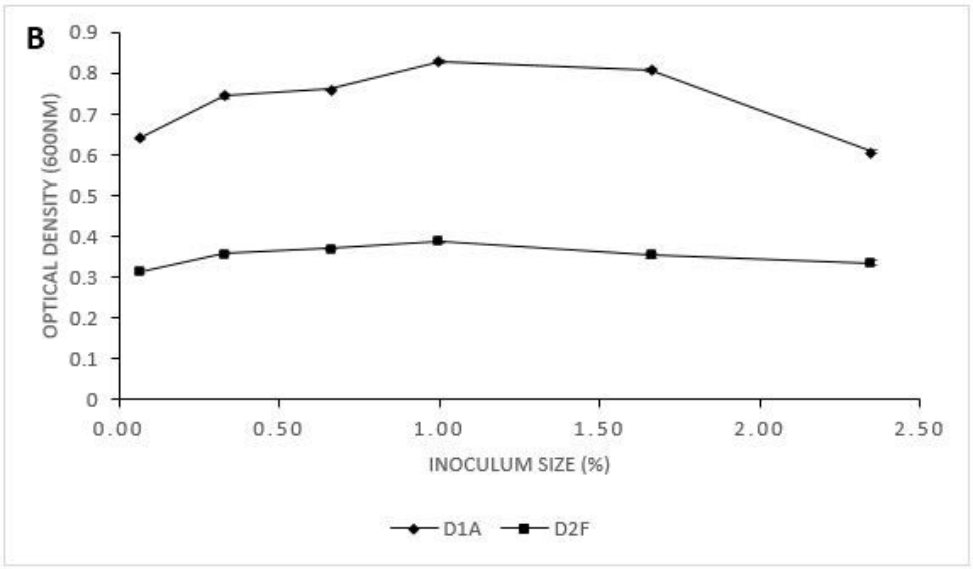
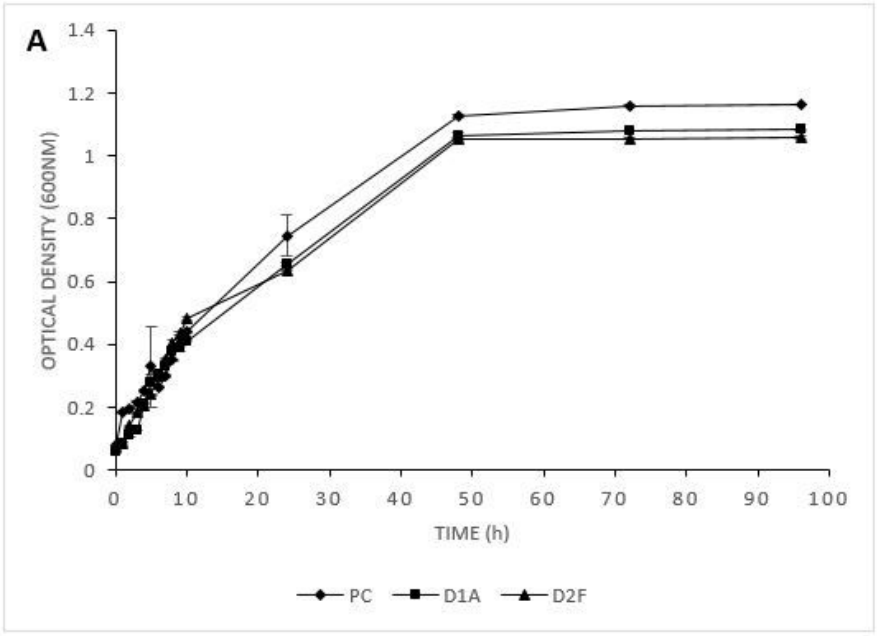


Figure 6

Effect of inoculum size on growth of bacterial strains (A) PC, D1A and D2F in the presence of ERY and (B) D1A and D2F in the presence of SXT.



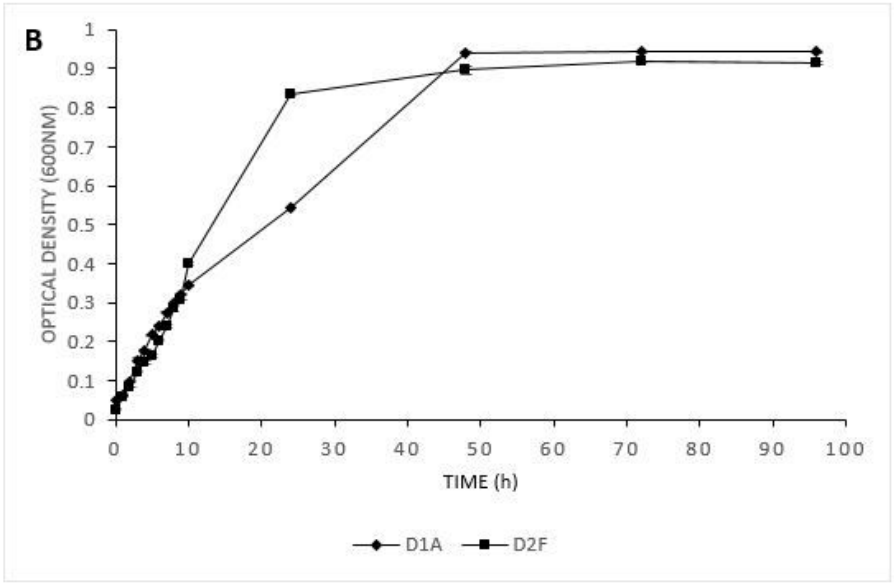


Figure 7

Growth curve analysis for bacterial strains (A) PC, D1A and D2F in the presence of ERY and (B) D1A and D2F in the presence of SXT

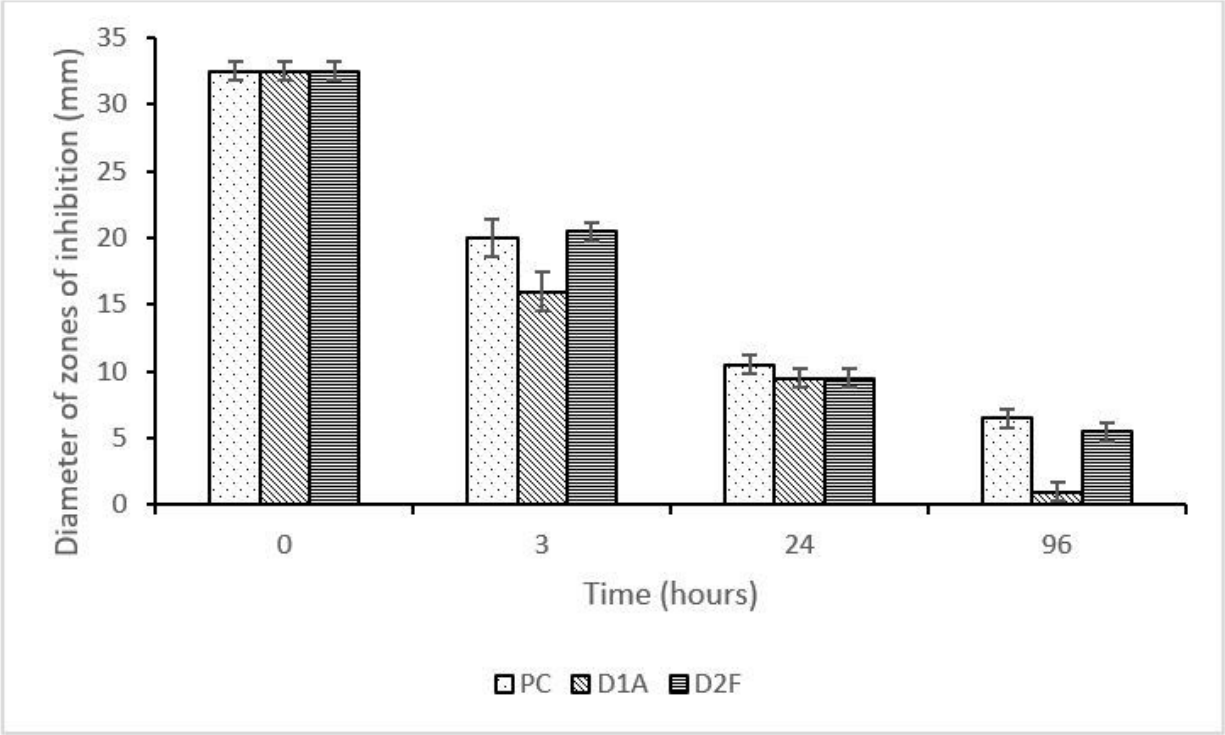


Figure 8

The antimicrobial potency of ERY during biotransformation by isolates PC, D1A and D2F at time intervals as measured using zones of inhibition.

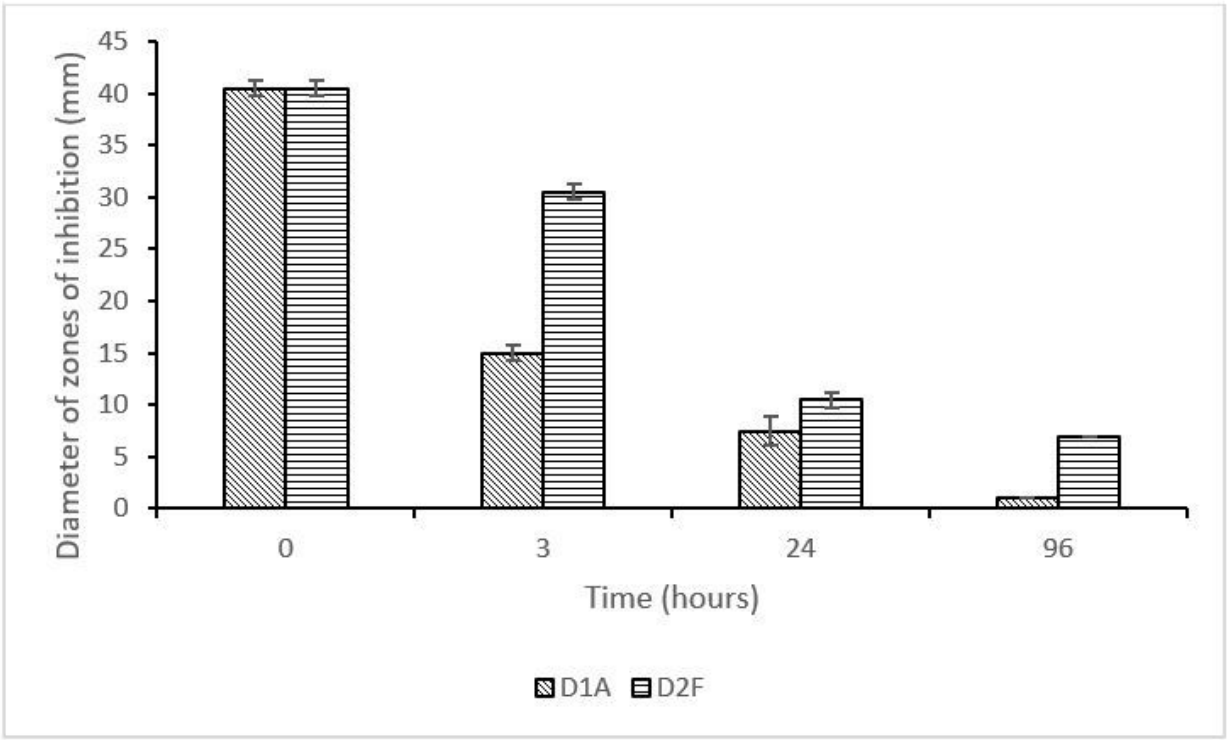


Figure 9

The antimicrobial potency of SXT during biotransformation by isolates D1A and D2F at time intervals as measured using zones of inhibition.