https://dx.doi.org/10.22161/ijmpd.2.3.1 *ISSN: 2456-8015*

Removal of Ciprofloxacin (CIP) by bacteria isolated from hospital effluent water and identification of degradation pathways

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Abstract— *Most antibiotics are metabolized incompletely by patients after administration and enter the municipal sewage with the patients' excretion. Therefore, studies on the biodegradability of some clinically important drugs can be taken as a very first step of an environmental risk assessment. The present study reports the biodegradation of CIP by Lactobacillus gesseri, Enterobacter sp., Bacillus sp., Bacillus subtilius and Micrococcus luteus which were isolated as CIP resistance, non pathogenic bacteria. The presence of antibiotic-resistant bacteria was identified using the 16s rRNA sequencing. A 0.5ml of overnight starved bacterial suspensions was introduced into medium containing CIP at 5 ppm. Triplicate samples were incubated at 28^0C with shaking at 100ppm. A 0.5 ml of subsamples was removed at 2 days interval for a period of 14 days. Samples were subjected to High Performance Liquid Chromatography (HPLC) analysis. Fourier Transform Infrared Spectroscopy (FTIR) analyses were carried out for each sample at the end of the 14 days to find structures of by-products. Complete degradation of CIP by L. gasserri was detected at the end of 14 days of incubation with average degradation rate of 0.182 ±0.15µg /day. Descending degradation rates were followed by Enterobacter sp. (0.75 ±0.03 d^{-1}) and Bacillus sp. (0.41±0.02d^{-1}) at 8 and 6 days respectively. However, clear cut degradation of CIP was not detected for B.subtilis and Micrococcus luteus respectively. Further, FTIR spectrum revealed that incubation of L. gesseri, Enterobacter sp. and Bacillus sp., changed the piperazine ring and quinolone part in the CIP structure while degradation occurred.*

Keywords— Ciprofloxacin, Degradation, Fourier Transform Infrared Spectroscopy (FTIR), High Performance Liquid Chromatography (HPLC), Lactobacillus gesseri.

I. INTRODUCTION

Antibiotics are designed to be refractory to biodegradation and to act effectively even at low doses (Sarmah *et al*., 2006). Since recently, the concern about potential ecological impacts of synthetic antibiotics has been increasing, because they may inhibit key environmental processes of nutrient regeneration, carbon and nitrogen cycles and pollutant degradation mediated by microorganisms (Rajilic-stojanovic *et al*., 2013). However, since mid-1990s, usage of these compounds was widespread and new analytical technologies were developed to quantify the amount of antibiotics (Lissemore *et al*., 2006).

The introduction of these compounds into the environment through anthropogenic sources which can constitute a potential risk for aquatic and terrestrial organisms was documented (Kummerer, 2009).

Quinolones represent a highly potent group of modern antibiotics, which were initially employed in the treatment of urinary tract infections in humans and animals (Avarin *et al*., 2013). They are very effective against gram negative bacteria via inhibition of the DNA gyrase responsible for preservation of DNA. The 6- fluorinated piperazinyl derivatives namely fluoroquinolones, are a more effective second generation of this family (Avarin *et al*., 2013). At present, they are broadly used for treatment for a great variety of respiratory diseases and enteric bacterial infections in human and food producing animals (Shaheen *et al*., 2013). The elimination of fluoroquinolones mainly occurs as parent compounds and as a consequence, significant quantities of the active ingredient are transported to the environment (Shaheen *et al.*, 2013). The most widely prescribed fluoroquinolone antibiotic is ciprofloxacillin (CIP), which is active against a broad spectrum of disease causing Gram negative and Gram positive bacteria

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(Sahinturak *et al*., 2016). In fact, the occurrence of CIP in the output of a treatment water plant, in hospital wastewater and in surface waters has been reported (Shaheen *et al*., 2013).

The physico-chemical properties of antibiotic (eg:- molecular structure, size and shape) will define their distribution in the environmental matrices (solids or water). Most conventional water treatment processes are not designed for the treatment of wastewater containing highly polar contaminats such as detergents and pharmaceuticals (Bergland *et al*., 2014). Therefore, practical and economical solutions must be achieved in order to reduce the daily amounts of antibiotics discharged into the environment. A wide range of chemical and physical methodologies can be employed for the removal of antibiotics (Bergland *et al*., 2014) where methods such as absorption, incineration, oxidation-reduction, photolysis, hydrolysis, reverse osmosis and chemical degradation are available at present for removing antibiotics from waste water (Ding *et al*., 2016). However, the real applicability of such techniques are expensive and inaccessible in most part of the world, especially in developing countries.

Bioremediation is an economically visible, cost effective green technology which may lead to degradation of antibiotics that produce simple compounds such as carbon dioxide, water nitrogen and organic materials during the microbial degradation processes (Li & Shang., 2010; (Sturini *et al*., 2012).

Therefore, many number of scientific reports showed promising environment-friendly antibiotic degradation methods using native aquatic bacteria. Maki et *al*., (2006) has isolated *Flavobacterium* strains responsible for the degradation of a group of antibiotics including OTC and AMP where recently Liyanage & Manage., (2015) have reported AMX and SUF degradation by the bacterium *B. cereus*. In the case of CIP, the available information is limited regarding bacterial degradation '*in situ'* as well as '*in vitro*'.

Thus, the aims of the present study were to isolate the CIP degradation bacteria and study the degradation kinetics of CIP along with determination of structural changes during the degradation pathways. Further, Ciprofloxacillin was selected to represent flouroquinolones, because they are the most widely used antibiotic at present in most part of the world.

II. MATERIALS AND METHODS

Chemicals and reagents

CIP standards (98%), HPLC and Bacteriological grade chemicals were purchased from Sigma Aldrich, USA.

Sample collection and enrichment study

Triplicate effluent water samples were collected from 75 sampling locations: including teaching hospitals, general hospitals, base hospitals and divisional hospitals. 50ml of effluent water from each sampling sites were enriched spiking, CIP antibiotic at final concentration of 60ppm in 100 ml erlenmeyer flasks and the final volume was topped up to 100 ml with sterile water and then the flasks were subjected to incubate at 28^0C ± 1 with 100rpm for 14 days in a shaking incubator [Liyanage & Manage., 2016 (a) and (b)]

After 14 days of incubation, standard pour plate method was carried out to isolate CIP resistance bacteria. LB medium which contained 60ppm of CIP was used to isolate CIP resistant (r) bacteria (CIPr) [Mulaudzi *et al*., 2011; Liyanage & Manage., 2016 (a) and (b)].

After three days of incubation at 28^0C, bacteria colonies with different morphological characters were picked up and re-suspended in sterilized liquid LB medium. Subsequently pure bacterial cultures were sub cultured and stored in agar slants at -20 ^{0}C in LB-glycerol media for further studies and identification (Manage *et al*., 2009; Liyanage & Manage., 2015)

Identification of CIP resistance bacteria

A total volume of 200µL of gDNA product was sent to Macrogen, Korea for sequencing. For identification of bacteria, DNA sequences were analyzed by the Basic Local Alignment Search Tool at the National Center for Biotechnology Information website (NCBI, http://www. ncb i. nlm. nih. go v/).

Screening of CIP degrading bacteria

The identified non pathogenic CIP resistance bacteria were transferred into 5 ml of liquid LB medium and incubated overnight at 28^0C. Then followed centrifugation to remove carbon source from the LB media and then the bacteria suspension was subjected to the starvation procedure, in phosphate buffer solution (PBS). Thereafter, equalized the turbidities of the bacteria suspension at A 590 = 0.35 (Manage *et al*., 2009).

150 µl of equalized bacterial suspension was inoculated into micro wells of the MT2 plates. The oxidization of the

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analyte (CIP) would result in a colour reaction taking place in the well by the reduction of tetrazolium dye which can be measured and quantified spectroscopically (Garland & Mills, 1991). Each bacterial strain (150µl) was tested against 5 ppm of CIP in triplicates. The control wells in the BIOLOG MT2 plate contained CIP and sterile PBS in triplicates. Then the plate was wrapped with wet tissue and incubated at 28 ^{0}C. The absorbance was measured by ELISA plate reader at 0, 12, 18, 24 and 48 hours interval at 595nm. The results were used to screen potential bacteria for CIP degradation (Naslund *et al*., 2008; Liyanage & Manage 2016.b).

Degradation kinetics of the isolated bacteria

Among 22 bacterial isolates, five bacterial strains namely *Lactobacillus gesseri*, *Enterobacter* sp., *Bacillus* sp., *Bacillus subtilius* and *Micrococcus luteus* showed high absorbance values for the BIOLOG MT2 plate assay. Further selection for degradation kinetics studies were based on the results obtained from the BIOLOG MT2 plate assay. A 0.5 µl of equalized bacterial suspension was inoculated into filter-sterile freshwater, containing CIP at a final concentration of 5 ppm respectively. All flasks were incubated at 28°C with continuously shaking at 100 rpm. A 0.5ml of sub samples were collected at two days intervals for a period of 14 days. Then the subsamples were centrifuged (12000 rpm) and supernatant of each sample was subjected to immediate freezing and stored at -20°C. Then the frozen samples were freeze-dried and reconstituted in 1 ml of 100% aqueous HPLC grade methanol and subjected to the HPLC analysis. Control samples were prepared in triplicates following same protocol without bacteria inoculation (Liyanage & Manage., 2014). The degradation rate (h) of CIP by bacteria was calculated according to the equation given bellow,

$h = \ln (C / C_0)/t$

where C_0 and C are the concentrations of CIP at the beginning and at the end of the time interval t, respectively (Manage *et al*., 2000).

Analysis of CIP

Analysis of antibiotics was carried out by using the HPLC system consisting of Agilent 1200 series following the modified method of Fernandez-Torres *et al*. (2010). The injected volume was 20µl and chromatography was performed at 30^{0}C. The mobile phase consisted of a mixture of 0.1% Glacial acetic acid in water (Component A): 0.1% Glacial acetic acid in methanol (Component B), 55:45 (v/v) was pumped in beginning at a flow rate of 0.7 ml/min. Concentrations of CIP was determined by calibration of the peak areas in UV detection range (230nm) with an external standard. The HPLC method has a detection limit of 0.5 µg/ml. CIP recovery was obtained greater than 90% with a relative precision of 15% (Perez burgus *et al*., 2012)

The concentration of CIP was analyzed using the equations given below. The peak area of the sample, (peak area of CIP = A_{CIP}), and the slope and intercept of each calibration curve was used to calculate the concentration of antibiotic in unknown samples (concentration of CIP =C_{CIP}). $C_{CIP} = (A_{CIP} – 10.451) / 1.5672$

FTIR analysis

The structural analysis of degradation product was obtained from FTIR in each sample at the end of 14 days of incubation (Wackerlig, & Schirhagl., 2015).

III. RESULTS AND DISCUSSION

Quinolone antibacterial drugs have many characteristics, such as broad antibacterial spectrum, high bactericidal activity, low toxicity and unique mechanism. Norfloxacin, enoxacin, ciprofloxacilin and ofloxacin are the representatives of the third generation antibiotics and have wide antibacterial spectrum (Luo et al., 2011).

Based on 16S rRNA gene sequence analysis with morphological characteristics and biochemical tests, the CIP degrading bacterial strains were identified as *Enterobacter* sp. (KM504128), *Lactobacillus gasseri* (KM4055978), *Bacillus* sp. (KM504129), *B. subtilis* and *Micrococcus luteus* respectively.

Due to an incomplete elimination, antibiotics have been introduced to the environment and their residues have been found both in waste and urban waters. Mathew and Unnikrishnan, (2012), reported that the CIP concentrations in the effluents waste water treatment plant was up to 31mg/l in pharmaceutical industries in India. During the wastewater treatment, 80 -90% of CIP is removed via sorption to sludge, which stabilizes the substance (Mathew and Unnikrishnan, 2012). Golet et al., (2003) found that 83±14% of CIP in the anaerobically digested sludge and may have resulted in the accumulation of antibiotics in the bio-solids treated soil.

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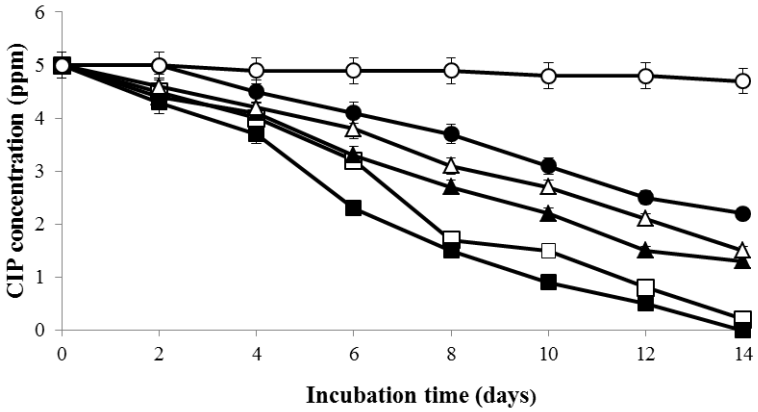

Fig.1: Degradation of CIP at 5 ppm .When error bars are not shown, standard deviation was less than the width of symbol (Open square; Enterobacter sp., Closed square; L. gasseri, Closed triangle; Bacillus sp., Open triangle; B. subtilis; Closed circle; M. luteus; Open circle; control)

Table.1: Degradation rates of CIP concentration in 5 ppm along with incubation time (given as mean value of triplicates)

Incubation time	**Degradation rate (d^{-1})**					
	Enterobacter s p.	*L. gasseri*	*Bacillus* sp.	*B. subtilis*	*M. luteus*	Control
0	0	0	0	0	0	0
2	0.25 ±0.08	0.35±0.07	0.30±0.11	0.20±0.12	0	0
4	0.25±0.06	0.30±0.04	0.15±0.01	0.20±0.01	0.25±0.01	0.05±0.00
6	0.40 ±0.02	0.70±0.01	0.41±0.02	0.20±0.01	0.20±0.01	0
8	0.75 ±0.03	0.41±0.00	0.31±0.03	0.35±0.02	0.20±0.02	0
10	0.10±0.04	0.33±0.01	0.20±0.00	0.20±0.01	0.30±0.01	0.05±0.00
12	0.35± 0.02	0.21±0.01	0.35±0.04	0.35±0.01	0.30±0.01	0
14	0.30±0.03	0.25±0.02	0.15±0.06	0.35±0.03	0.15±0.03	0.05±0.00

Fig.1 shows the degradation of CIP by *Enterobacter* sp. (KM504128), *L. gasseri* (KM4055978), *Bacillus* sp. (KM504129), *B. subtilis* and *M. luteus* during 14 days of incubation.

Most of the antibiotic compounds have shown low biodegradable activity (Luo et al., 2011). Similar to other antibiotics, CIP is not readily biodegradable and it also strongly sorbs to soil. At present study, CIP was completely removed within 14 days of incubation by *L. gasseri* at average rate of 0.182 ±0.15µg/day. CIP degradation by *M. luteus* was initiated following 2 days of lag period and gradually increased afterwards achieving 56% of degradation at 14 days of incubation. *Enterobacter* sp., *Bacillus* sp., *B. subtilis* showed 96%, 74%, 70% removal of CIP at the end of 14 days of incubation and the degradation was initiated without a lag phase (figure 1). Comparison of CIP removal patterns; among five bacterial strains *L. gasseri* is a most potent candidate for rapid removal of CIP with a steep slope. *Enterobacter* sp. also showed the highest degradation rate (0.75 ±0.03 d^{-1}) after eight days of incubation where *L. gasseri* (0.70±0.01d^{-1}) and *Bacillus* sp. (0.41±0.02d^{-1}) showed their maximum degradation rate at six days of incubation. However, a clear-cut degradation was not observed in the cultures of *B.subtilis* , *M. luteus* even at 14 days of incubation (Table 1).

Analysis of the sterile controls showed no significant loss of

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CIP during the incubation and only 6% of decrease was detected. This may be due to natural degradation and photodegrdation effect.

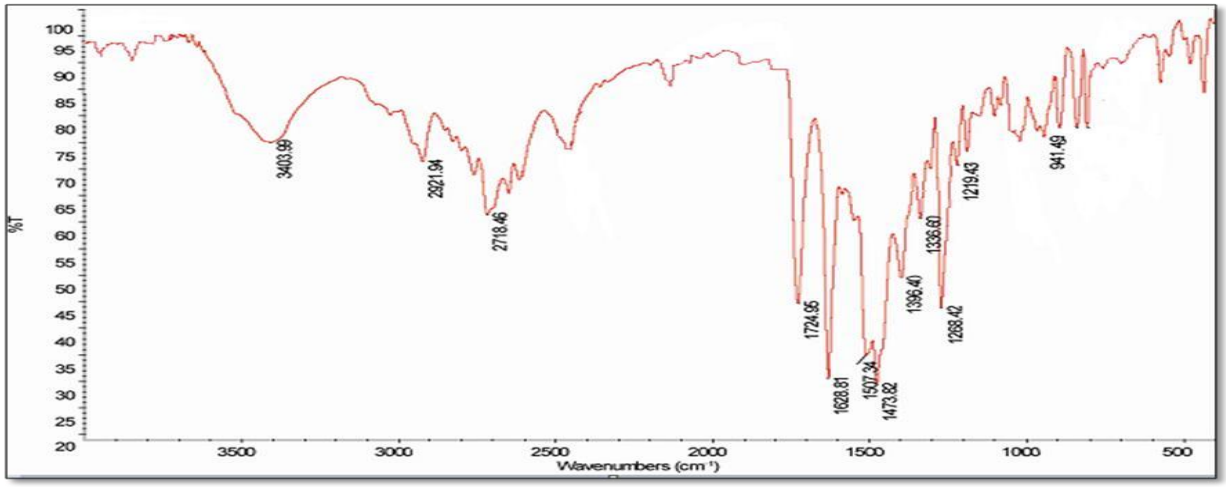
According to Golet et al., (2003) in soil until day 6, the degradation rate was relatively high (around 0.03 day-^{1}), thereafter, it was low but constant (0.008 day-^{1}). The accumulation of CIP lead to development of CIP resistance bacteria which caused a health risk to both humans and animals (Nasland et al., 2008). Therefore, findings of the degradation processes are important in order to decrease the CIP contaminant amount in the waste water before it reached the natural environment.

Once fluoroquinolones (eg: CIP) reach the aquatic environment, they are exposed to multiple environmental factors and may undergo various degradation processes. Limited number of research studies have found that some fungus and bacterial species could significantly transform CIP but at relatively low rates (Prieto et al., 2011; Parshikov et al., 2001). Prieto et al., (2011) reported that white rot fungi and brown rot fungi degraded more than 60% of CIP in tested samples.

Many previous studies reported low biodegradation rates for antibiotics in their findings they degraded more than 50% of CIP from the samples (Kummerer et al., 2000; Rajilic-stojanovic et al., 2013). In the present study *L. gasseri. M. luteus, Enterobacter* sp., *Bacillus* sp. and *B. subtilius* were identified as potent CIP degradares. Unlike *L. gasseri (100%) and M. luteus (96%); Enterobacter* sp., *Bacillus* sp. and *B. subtilis (*figure 1*)* are unable to degrade the complex cyclic structure of CIP more than 75% and this has resulted in retarded degradation rates for CIP. Presence of benzene ring and piperazinyl ring in CIP could be the possible cause for the failure of complete removal of the antibiotic. This was slightly different from the amoxicillin degradability of the species in same genera. According to Liyanage and Manage (2015), *B. cereus* removed amoxicillin (AMX) completely from the samples after 8 days of incubation.

It was recorded that CIP mainly reduced the microbial activity at the beginning, because it is a biostatic compound that targets growing microorganisms (Vasconcelos et al., 2009; An et al.,

2010). Thus, in the present study, this may be the reason to get low degradation rates at the beginning and with the time, degradation rates increased (Table 1).



(a)

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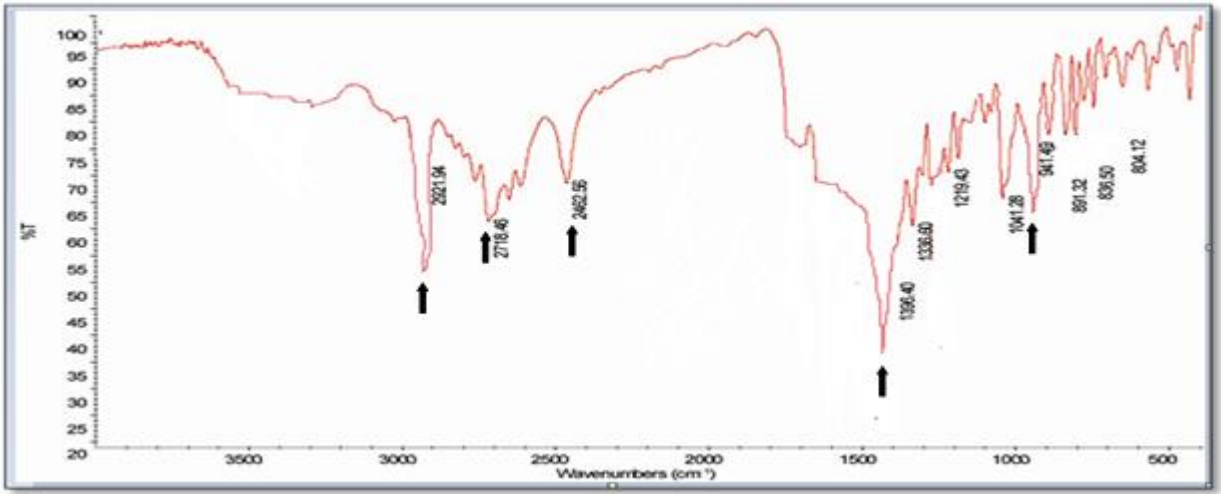
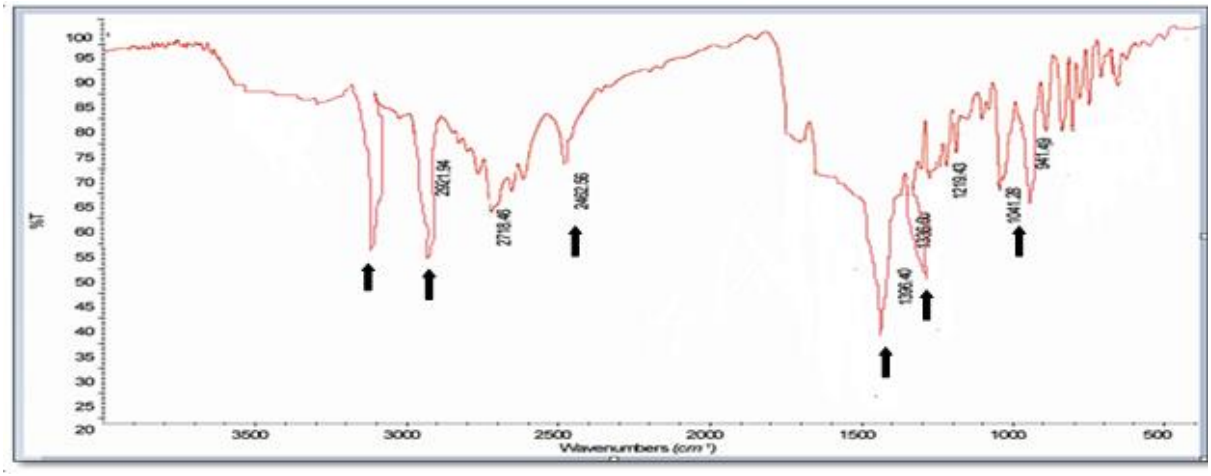
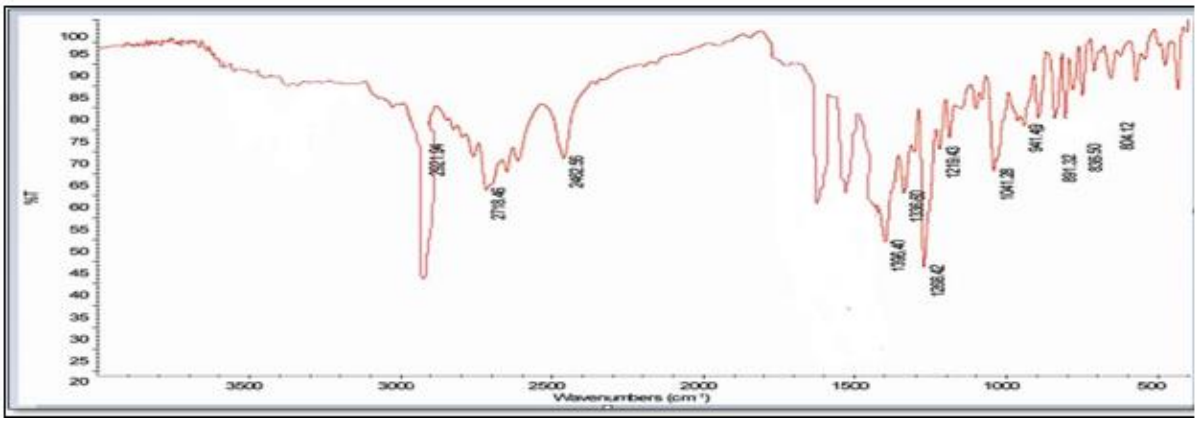


(b)



(c)



(d)

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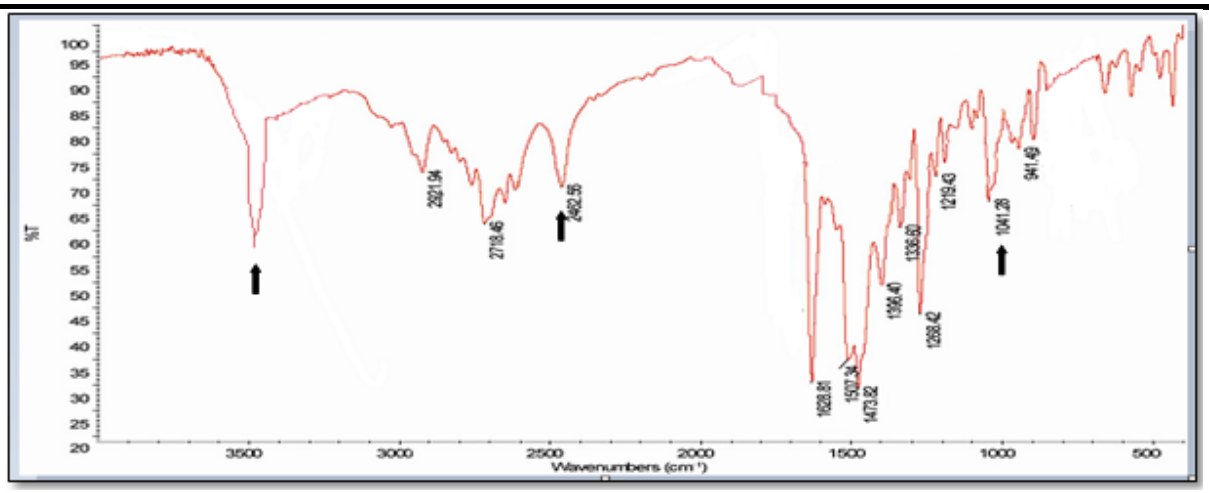
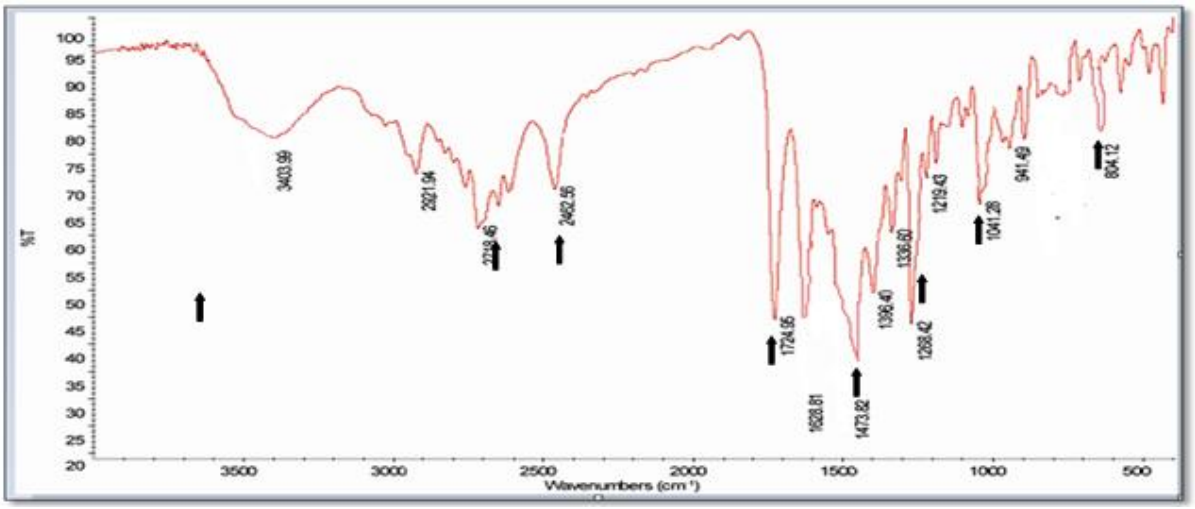


(e)



(f)

Fig.2: FTIR spectrums (a) Initial sample (0 day), (b) Treatment with L. gesseri at 14 days, (c) Treatment with Enterobacter sp. at 14 days, (d) Treatment with Bacillus sp. at 14 days, (e) Treatment with B. subtilis at 14 days, (f) Treatment with M. luteus at 14 days [black arrows showed changing of functional groups from initial spectrum]

The infrared spectra of samples at 0 day and at 14 days are shown in figure 2. Figure 2 (a) indicated the FTIR spectra for initial sample. Among the vibrations, those at 1473.82 cm^{-1}, 1507.34 cm^{-1} and 1628.81 cm^{-1} were assigned to vibration absorption of the CH_2 on the benzene ring. The 1724 cm^{-1} and 3403 cm^{-1} stretching vibrations are attributed to carbonyl and hydroxyl in carboxyl group respectively. The 1396 cm^{-1} and 941 cm^{-1} vibration correspond to the bending of O-H and around 800 cm^{-1} indicate the absorption peak of secondary amine. Overall, the spectrum is indicative of CIP, since it is similar to standard spectrum of CIP containing HCl (Kummar and Min., 2011; Tan et al., 2012; Yan et. al., 2012)

It can be observed in figure 2 (b) that the peaks in 3403 cm^{-1}, 1724 cm^{-1}, 1628 cm^{-1} disappears while intensity of peaks in 2921 cm^{-1}, 1396 cm^{-1} and 941 cm^{-1} increased compared to the initial spectrum, which accounts for bacteria degradation. Two new stretching absorption peaks

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of alkyene C (2462 cm^{-1}) and C-F (1041 cm^{-1}) were found which indicated the changes of quinolone group due to bacterial degradation (Kummar and Min., 2011; Tan et al., 2012; Yan et. al., 2012). Figure 2 (c) shows new stretching absorption peak of N-H which appears at around 3100 cm^{-1} indicating the changes of pyradizine ring whereas new peak at 1412 cm^{-1} (-COO) indicating the changes in quinolone ring. Other peak changes more similar to spectrum 2 (b) were found.

Compared with standard spectra, from figure 2 (d), the strong intensive peak was observed at 2921 cm^{-1} indicating the alkynene bonds and new peaks appeared at around 820 cm^{-1} which correspond to C≡N, C≡C and C=O groups [figure 2 (d)]. Those changes ensured the bacteria degradation of CIP (Kummar and Min., 2011; Tan et al., 2012; Yan et. al., 2012).

The FTIR spectrums (figure 2.e & f) obtained for *B. subtilis* and *M. luteus* were different from spectrums of figure 2 (b)-2 (d) whereas less or more similar to initial spectrum [figure 2(a)]. The vibrations correspond to bonds in benzene ring (1472 cm^{-1}, 1628 cm^{-1}) and pyrazene ring still appeared in spetrums of *B. subtilis* and *M. luteus* following 14 days of incubation respectively (Kummar and Min., 2011; Tan et al., 2012; Yan et. al., 2012).

Thus, from the results it can be explained that two different degradation processes took place among those 5 bacterial strains. *L. gesseri*, *Enterobacter* sp., *Bacillus* sp. were shown similar degradation pattern compared to other bacterium *B. subtilis and M. luteus*. *L. gesseri*, *Enterobacter* sp. and *Bacillus sp.* changed the structure of pyrazine ring and quinolone ring while, other two bacteria only reduced the quantity of antibiotic with less structural changes during the incubation process.

Regarding the fluoroquinolones, several studies on pure water solutions revealed that photodegrdation takes place mainly via substitution on the piperazinyl ring in the position 7 (figure 1) (Paul et al., 2010) where, additional experiments revealed that the presence of organic and microbiological matter in water solution significantly affects the degradation of fluroquinolones (eg: CIP) (Parshikov et al., 1999; Paul et al., 2010). The compounds containing a β-lactam ring (cefotian hydrochloride, penicillin G and etc) could have been degraded chemically by hydrolactic ring opening without oxygen consumption. Thus, it was clear that the most common degradation pathways for the CIP, may be mediated by hydroxyl radical attack.

It is known that the degradation of CIP occurs at least at two functionalities of the CIP molecule; the piperazinyl substituents and quinolone moiety (Lissemore et al., 2006; Dewitte et al., 2008 Santoke et al., 2009; Wei et al., 2013) The obtained spectrums (figure 2) were compared to those previously reported results in the literature, and the spectrums correspond for bacterium *L. gesseri*, *Enterobacter* sp. and *Bacillus* sp. were found to be very similar to CIP degradation products recorded by others' studies (Prieto et al., 2011; Parshikov et al., 2001). These compounds correspond to the partial and complete elimination of the piperazynilic ring of the ciprofloxacin molecule which were found in the present study (Figure 2 (b), (c), (d)).

The decrease of the vibrations that correspond to piperazine ring compared with initial FTIR spectrum of CIP (figure 2 (a)] suggest that multiple sites on the piperazine ring were oxidized and potentially forming carboxy groups. Guo et al., (2013), recorded in their experiment that the antibacterial activity in the sample was generally decreased by the bacteriological treatment and the products of the quinolone ring structure remained. These results also suggested that the cleavage of the piperazinyl ring is capable of eliminating the antibacterial activity of CIP. However, complementary analytical techniques, such as nuclear magnetic resonance (NMR), and/or use of authentic standards are needed in future work to further confirm the structure of CIP transformation product.

IV. CONCLUSION

As one of the third-generation antimicrobial quinolones, ciprofloxacilin, possessing broad – spectrum of antimicrobial activity, wide application in clinical treatment of infections and diseases caused by gram negative and positive bacteria. *L. gasseri efficiently* degraded (100%) CIP resulting in a complete removal of CIP from the sample while *Enterobacter* sp., *Bacillus* sp., *B. subtilius*, *M. luteus* degraded 96%, 74%, 70%, 56% respectively. The FTIR spectrums of each bacterium showed the changes in antibacterial active sites (piperazine ring and quinolone part) of CIP and confirmed the degradation of CIP by the bacteria isolated. Therefore, it can be concluded that the five bacterial strains are the potent candidates that can be introduced into waste water effluents to remove CIP in effluent water before reaching natural environment.

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ACKNOWLEDGEMENT

The authors wish to thank the University of Sri Jayewardenepura in Sri Lanka for providing the financial support for the study (ASP/06/RE/SCI/2013/20; Centre for water quality and algae research).

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