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Bioremediation of Acetaminophen Mediated by *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104 (T)

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Acetaminophen is one of the widely used over-the-counter drugs. Its residual concentration has been detected in pharmaceutical industry wastewater, effluent treatment plant and surface water. Eco-toxicological effects of paracetamol have been reported on seed germination, fish and algae. Drug resistance in pathogenic micro-organisms has been attributed to the overuse or continuous exposure of pathogens to drugs. The work was conducted from February 2022 to July 2022. Current study was carried out on the acetaminophen (paracetamol) degradation potential of *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T) which was isolated from effluent of pharma industry. Identification was done using 16S rRNA sequencing technique. Isolate was found to degrade 82.8% of acetaminophen (2500 ppm) after five days of incubation. Based on the analytical techniques high resolution mass spectrometry (HRMS) and proton nuclear magnetic spectroscopy (1H NMR) study, 4-aminophenol was found to be biodegradative metabolite. *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T) can degrade paracetamol through biological approach. Compared to paracetamol, its biodegradative product 4-aminophenol has more toxicity against algae. The paracetamol biodegradation potential of *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T) was studied and could be used as microbial candidate for remediation of paracetamol contaminated sites.

KEYWORDS

Acetaminophen, Biodegradation, Pathogens, Phylogenetics, Wastewater

1. INTRODUCTION

With the emergence of diseases and multidrug resistant properties in micro-organisms, drug usage has increased. Pharmaceutical industrial wastewater is a mixture of antibiotics, raw materials, surfactants, variety of medicines and cosmetic products [1]. Release of pharma micropollutants in the aquatic environment is attributed to domestic, industrial and hospital activities [2,3]. In Indian environmental matrices, besides pesticides and persistent organic pollutants, pharmaceutical residues have been considered as developing environmental contaminant due to their negative impact on aquatic life [4]. Occurrence of pharmaceutical drugs and personal care products in water is mainly influenced by type, seasonal variation, concentration, etc. In Ganges river, fifteen pharmaceuticals and personal care products (PPCPs) were detected in which caffeine, tetracycline, acetaminophen and triclosan were the most abundant compounds [5]. Cholesterol lowering drug (clofibric acid), analgesics (diclofenac), psychiatric drugs (diazepam, carbamazepine, nordiazepam) and antibiotics has been reported in treated wastewater of Europe, Asia and United States [6]. During pandemic period of SARS Covid-19, consumption of hydroxychloroquine and paracetamol was increased based on wastewater epidemiological studies [7]. Persistence of drug in environment is attributed to their high solubility and little biodegradability which could affect microbial community [8]. Discharge of drugs into environment is leading to development of antimicrobial resistance (AMR) in pathogens *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* Enterobacter collectively called 'ESKAPE pathogens' [9].

Release of drugs into environment is causing adverse effects on non-target organisms, like bacteria, algae

Table 1. *Occurrence of paracetamol in water*

Location	Water type	Paracetamol conc.	Reference
Kuwait	Wastewater	0.101-20.86 μg/L	[27]
United Kingdom	Tyne river	65 μg/L	[28]
France	Well supplying drinking water	0. 211 μg/L	[6]
India	Yamuna river	0.157-1.708 μg/L	[29]
	Ganga river	6.81-247 μg/L	[5]
	Sewage treatment plant Udupi	9 μg/L	[30]
	Nag and Pili rivers, influent and effluent of sewage treatment plant	11.50 μg/L	[31]
California	Public drinking water supply	1.89 μg/L	[32]
Australia	73 river sites	7150 μg/L	[33]
Kenya	Nairobi river basin	10-30 μg/L	[34]
South Africa	Darvill wastewater treatment plant	2.97-5.96 μg/L	[35]
	Msunduzi river	1.74 μg/L	
Germany	Elbe river	0.066 μg/L	[36]
Southern France	Lergue river	0.211 μg/L	[6]
Europe	Sewage effluents	6 μg/L	[37]
United States of America	Natural water	10 μg/L	[38]
Serbia	Surface waters	79.2 μg/L	[39]

and fish. Paracetamol has been reported to exert deleterious irreversible effects on non-target organisms even at low levels of exposure [10]. Eco-toxicological studies on paracetamol (25, 50, 75, 100, 125 and 150 mg/L) using *Nostoc muscorum* cyanobacteria were conducted where paracetamol was found to enhance oxidative stress with EC50 (113.68 mg/L) after 6th day of treatment [11]. Edible plant spinach was reported to have adverse effect on photosynthesis after exposure to paracetamol concentration in range of 50-200 ppm which gets bioaccumulated in aerial parts on 4th day. In the same experiment, spinach root colonisers Burkhulderia, Sphingomonas, Pseudomonas, Staphylococcus, Stenotrophomonas and Kocuria were found to have paracetamol biodegradation potential after 8 days [12]. Paracetamol has been reported to induce stress in plants like *Lemna minor* and *Lactuca sativa* [13,14].

Paracetamol (acetaminophen; N-acetyl-para-aminophenol) is one of widely used analgesic and antipyretic drugs which is emerging as pharma pollutant worldwide [15]. The Environment Agency (EA) of England and Wales proposed ranking system with list of top 10 compounds with potential risk of pollution in aquatic environment, where paracetamol has been classified in 5th place [16]. Occurrence of paracetamol in water is given in table 1. There are reports on degradation of paracetamol using physico-chemical processes (advanced oxidation, membrane filtration, activated carbon, ozonation, TiO_2 photolysis, electrochemical treatment, Fenton reactions and chlorination) and biological processes (aerobic and anaerobic degradation, membrane bioreactor and phytoremediation) [17]. Biodegradation of pharma micropollutants, like paracetamol has gained interest among researchers due to its low-cost and efficacy. Bacteria, namely *Rhodococcus ruber*, Stenotrophomonas, Pseudomonas, *Delftia tsuruhatensis, Cunninghamella echinulate,* Shinella have paracetamol biodegradative potential [18-22]. Paracetamol biodegradation pathway involves catalytic activity of aryl acylamidase, deaminase and hydroquinone 1,2-dioxygenase yielding 4-aminophenol and hydroquinone as end products [23-26].

2 MATERIAL AND METHOD

2.1 Chemicals

Chemicals (acetaminophen) and reagents (methanol,

water) used for bacterial cultivation were of analytical grade and purchased from Loba Chemicals. The Bushnell Haas medium (g/L) comprised of magnesium sulphate (0.2), calcium chloride (0.02), potassium dihydrogen phosphate (1.0), dipotassium hydrogen phosphate (1.0), ammonium nitrate (1.0) and ferric chloride (0.05). The pH of the medium was 7.0 ± 0.2.

2.2 Isolation and identification of micro-organisms

Effluent wastewater sample was collected from the site of industrial discharge. The collected sample was stored at 4°C until use and then it was used for the isolation of acetaminophen tolerating bacteria (with conc. 2500 ppm) [15]. The identification of isolates was done at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune, Maharashtra, India. Genomic DNA was isolated by the phenol/chloroform extraction method followed by PCR amplification of the 16S rRNA gene using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3'] [40]. The amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation and directly sequenced on ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster city, California) as per manufacturer's instructions. Essentially, sequencing was carried out from both ends using additional internal primers so that each position was read atleast twice. Assembly was carried out using Lasergene package followed by identification using the EzBioCloud database [41].

2.3 Acetaminophen degradation studies

After primary screening of the bacteria to tolerate 2500 ppm concentration of acetaminophen, degraation studies was carried out using Bushnell Haas medium with pH 7.0, 1% inoculum and 30°C temperature under the stationary conditions. After every 10 hr of incubation, biomass concentration was also determined as per dry weight method [42]. The pH of the cell free broth was determined using pH meter. Residual concentration of acetaminophen was determined as per protocol of Sane [43]. Acetaminophen colourimetric assay using Sane method is based on principle that at elevated temperature, acetaminophen reacts with sodium hydroxide which resulted in a crimson reaction product with an absorption maximum at 463 nm [43]. Briefly, 1 mL cell free supernatant was mixed in 1 mL of 10% sodium hydroxide. Mixture was kept in boiling water bath for 15 min, followed by addition of 1.5 mL water. After gentle mixing, the reaction mixture was incubated at room temperature for 100 min. After incubation, content was diluted upto 4 mL with water. Absorbance was taken at 463 nm using water and sterile broth as control. Standard dose response of acetaminophen (100-1000 μg/mL) was used for the determination of residual concentration of acetaminophen after every 10 hr of incubation. The degradation (R) of APAP was calculated by equation 1.

$$R\ (\%) = (C_0\text{-}C_t)/C_0 \times 100 \qquad ...(1)$$

Where C_0 is absorbance at initial concentration of APAP and C_t is absorbance after incubation at time 't'.

2.4 Characterization of biodegradative product

Analysis of paracetamol and its bacterial transformed product was determined using reverse phase HPLC method as per modified protocol of Zhang [44]. Five days old fermented broth was centrifuged at 10000 rpm for 30 min. Broth was concentrated and extracted with methanol. The organic phase was collected and evaporated using rota evaporator. The elution of paracetamol was done using reverse phase HPLC with isocratic mode, JASCO (250 × 4.6 mm, packed with 5 μ). The solvent system used was methanol:water (15:85) with 1 mL/min flow rate and monitored at 240 nm. Identification of degradative metabolite was done using high resolution mass spectrometry (HRMS). Bruker Impact HD instrument was used for analysis of mass of standard and its biodegradative metabolite extracted from fermented broth. A dual electrospray ionization source was operated in positive ion mode to acquire full scan mass spectra from 50-1200 m/z with a scan rate of 4.0 spectra/sec. The source gas temperature was set at 200°C and 7.01 L/min flow rate. Sample injection volume was 10 µL. Nebulizer pressure was adjusted to 1.7 bars. Data analysis was performed using Bruker Compass data analysis 4.2 software. Nuclear magnetic resonance (NMR) spectroscopic analysis proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian 500 NMR spectrometer operating at 300 MHz with DMSO as solvent. The sample temperature was stabilized at 25°C.

2.5 Algal inhibition assay

The *Chlorella vulgaris* culture was pre-adapted to the laboratory condition by growing algae in bold basal medium (BBM) with 10% inoculum (OD at 620 nm = 0.2) [45]. Culture was grown in 50 mL medium with 25-28°C temperature and 1500-2000 lux irradiance of

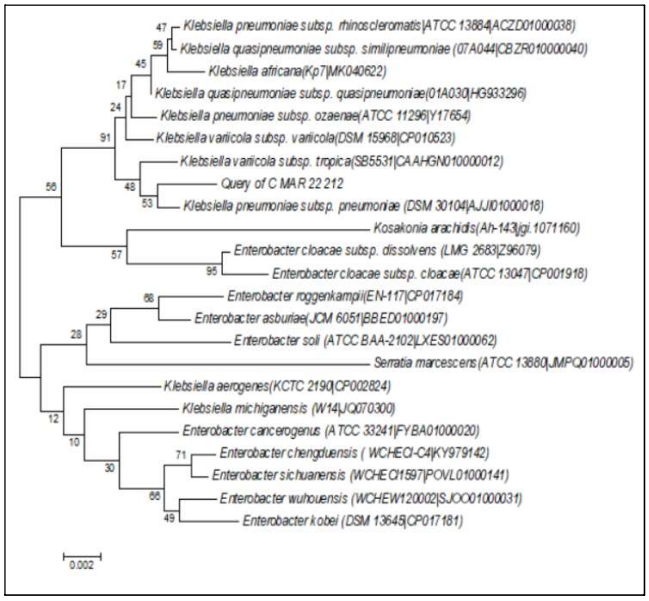

Figure 1. *Phylogenetic tree based on 16S rDNA sequence*

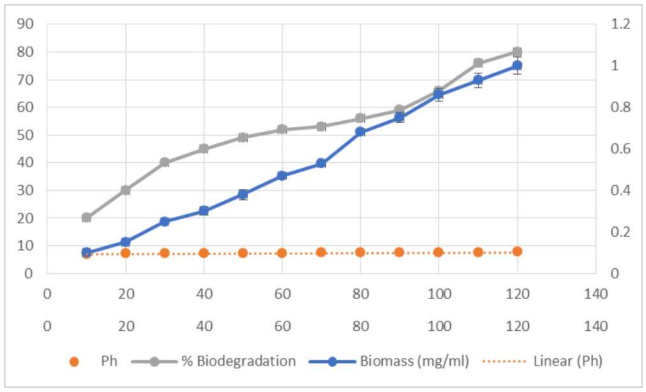

Figure 2. *Time course effect on the biodegradation studies of acetaminophen by Klebsiella sp*

light. Growth was monitored by measuring optical density at 620 nm as described by Wang [45]. Specific growth rate (μ) was calculated using the formula given below.

$$\mu\ (Day^{-1}) = (\ln N_2 - \ln N_1)/(t_2 - t_1) \qquad ...(2)$$

Where N_1 and N_2 are OD 620 nm values taken at times t_1 and t_2 within exponential phase.

For toxicity testing, in 45 mL of sterile BBM medium, 5 mL algal inoculum was inoculated. Range of testing substance paracetamol (0.01-1000 ppm) and 4-aminophenol (0.01-1 ppm) was determined based on experimentation. Incubation conditions were maintained at 25-28^{0}C temperature and 1500-2000 lux irradiance

Table 2. *Analysis details of the isolate*

Total number of sites for the analysis	1372
Conserved sites	1298
Variable site	72
Parsimony informative sites	52
Analysis	Phylogeny reconstruction
Statistical method	Neighbour-Joining
Test of phylogeny	Bootstrap method
No. of bootstrap replications	1000
Substitution model	Tajima-Nei method
Substitutions to include	Transitions + transversions
Rates among sites	Gamma distributed (G)
Pattern among lineages	Same (homogeneous)
Gaps/missing data treatment	Pairwise deletion

of light. After every 24 hr, optical density was determined at 620 nm using Systronics double beam spectrophotometer. EC50 values were determined after 96 hr of study using IC_{50} calculator [47].

3. RESULT AND DISCUSSION

3.1 Isolation and identification of micro-organism

The strain isolated from effluent sample was found to tolerate 2500 ppm concentration of acetaminophen without any effect on growth. The isolate was identified based on morphological and 16S rRNA sequencing. The isolate showed 99.56% similarity with *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T) (accession number : AJJI01000018). The evolutionary history was inferred using the neighbour-joining method [48]. The evolutionary relationships of taxa are shown in figure 1. The optimal tree with the sum of branch length = 0.11003849 is shown. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [49]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The analysis involved 23 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1372 positions in the final dataset (Table 2). Evolutionary analyses were

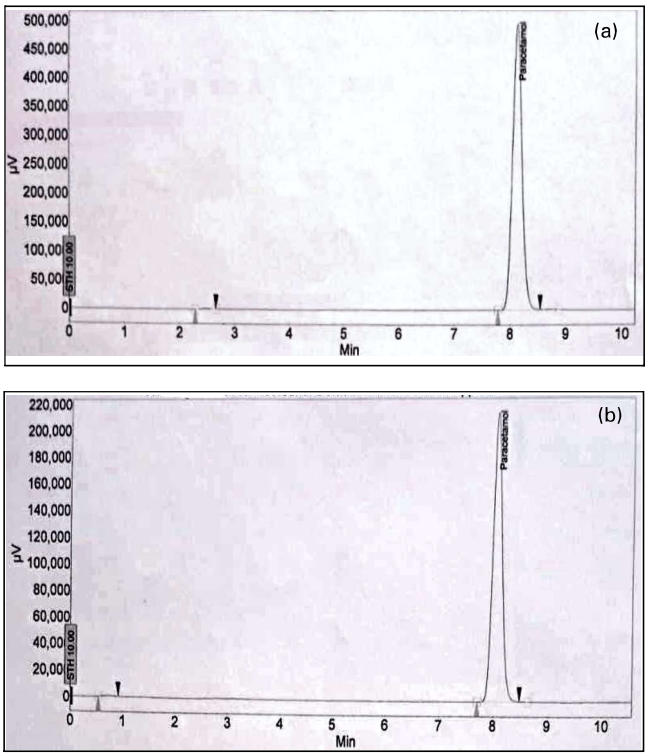

Figure 3. *(a) Reverse phase HPLC chromatogram of standard acetaminophen and (b) reverse phase HPLC chromatogram of methanolic extract of Klebsiella sp. (5th day BS fermented broth with 2500 ppm acetaminophen)*

conducted in MEGA6 [50].

3.2 Acetaminophen degradation studies

Klebsiella pneumoniae subsp. *pneumoniae* DSM 30104 (T) was inoculated in Bushnell Haas medium containing acetaminophen (2500 ppm) as sole carbon and energy source. Bushnell Haas is generally used for the bioremediation of xenobiotic compounds. Chopra has used Bushnell Haas medium for the degradation of acetaminophen using *Bacillus drentensis* strain S1 [15]. Biodegradation of acetaminophen was found to be maximum on 5th day of incubation when micro-organism was in late log phase. The time course effect of biodegradation of acetaminophen by *Klebsiella pneumoniae subsp. pneumoniae* DSM 30104(T) is represented in figure 2.

3.3 Characterization of biodegradative product

Reverse phase HPLC was carried out for quantitation of acetaminophen in fermented broth figure 3a. Two peaks were observed in HPLC chromatogram, one of paracetamol and second compound with retention time of 0.708 min. The biodegradation of acetaminophen

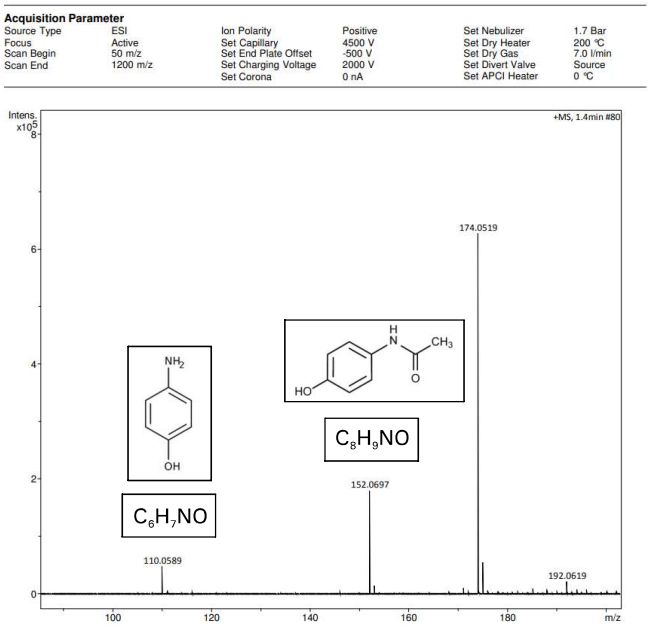

Figure 4. *HRMS spectra of degradative product of acetaminophen*

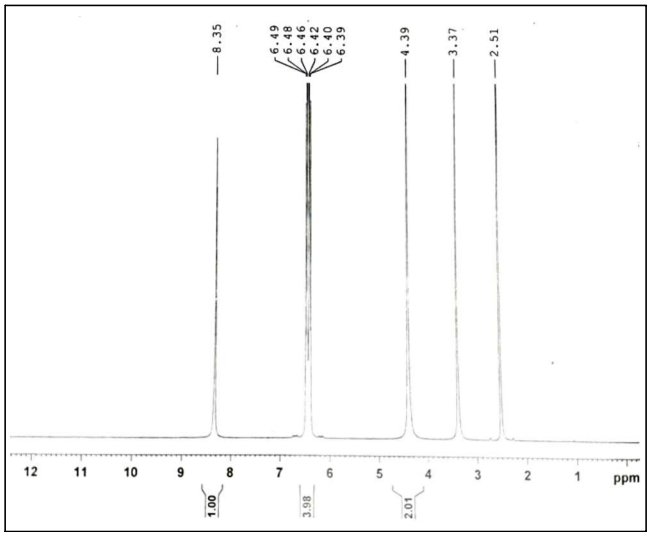

Figure 5. *1H-NMR spectra of acetaminophen degradative product (4-aminophenol) by Klebsiella sp.*

observed was 82.3% after five days of incubation. The chromatogram of standard acetaminophen (paracetamol) and methanolic extract of *Klebsiella sp.* 5th day BS fermented broth with 2500 ppm acetaminophen is shown in figure 3b. Two peaks were observed with 152.0697 and 110.0589 m/z corresponding to paracetamol ($C_8H_9NO_2$, molecular weight: 151.16) and 4-aminophenol (C_6H_7NO, molecular weight: 109.13) as given in figure 4. For structural confirmation of degradative metabolite, proton NMR was carried out.

Table 3. *List of paracetamol degraders with end product*

Microorganism	Acetaminophen concentration	Degradation (%)	Time for biodegradation (days)	Degradative product of paracetamol	Reference
Rhodococcus ruber	0.75-500 mg/mL	86	20	p-aminophenol, pyrocatechol and hydroquinone	[18]
Stenotrophomonas sp. F1 and *Pseudomonas sp.*	2000 mg/L	87.1	-	4-aminophenol and hydroquinone	[19]
Brevibacterium frigoritolerans, Corynebacterium nuruki and *Enterococcus faecium, Bacillus cereus* (200 ppm) of paracetamol	200 mg/L	97, 97 and 86.9	-	4-aminophenol, hydroquinone and 2-hexenoic acid	[51]
Flavobacterium, Dokdonella and Methylophilus	50 ppm in WWTP	-	2	4-aminophenol, hydroquinone and one unknown compound	[52]
Delftia tsuruhatensis, Pseudomonas aeruginosa, membrane bioreactor	100 μg/L	99.9	16	Hydroquinone	[53]
Pseudomonas sp. strain ST-1	-	76.8	3	-	[54]
Pseudomonas aeruginosa strain HJ1012	2200 mg/L	-	-	p-aminophenol and hydroquinone	[55]
Bacillus drentensis strain S1	50-1200 mg/L	94.5	2	Oxalic acid, 2-isopropyl-5-methylcyclohexanone and phenothiazine	[15]

1H-NMR (300 MHz, DMSO-D6) spectra of acetaminophen degradative metabolite by *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T) is given in figure 5 where chemical shifts δ (ppm) are : 8.35 (s, 1H), 6.49-6.39 (M, 6H) and 4.39 (s, 2H). Based on peak pattern, amino, hydroxyl group and benzene ring were analysed. NMR peak at 2.51 ppm belongs to solvent DMSO.

In this current study, the isolate *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T) was found to have the potential to degrade acetaminophen in concentration of 2500 ppm as sole source of carbon and energy. After five days of fermentation, the isolate was found to degrade 82.8% of acetaminophen and converted to 4-aminophenol. The confirmation of degradative metabolite was done using high resolution mass spectroscopy and proton nuclear magnetic spectroscopy. Data related to the microorganisms, concentration of acetaminophen and days required for the biodegradation with their end products is given in table 3. Compared to the reported studies, the isolate *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T) showed high tolerance and acetaminophen degradative potential with 82.8% degradation after five days of incubation. Analytical techniques like proton NMR and HRMS technique support formation of 4-aminophenol.

3.4 Algal inhibition assay

Algal inhibition assay was performed for the toxicity evaluation of paracetamol and its biodegradative product 4-aminophenol. IC_{50} value of paracetamol was found to be 461.5882 ppm which was higher as compared to the result of Wang [46]. For 4-aminophenol, the value of IC_{50} was found to be 0.3162 ppm which suggests more ecotoxicity of degradative product as compared to parent compound paracetamol.

3.5 Discussion

Drugs degrading bacteria have been reported in industrial discharge, hospital wastewater and effluent [55-59]. Reports are available on the bacterial tolerance of acetaminophen in the concentration of 50-2500 ppm [44,52]. Acetaminophen (paracetamol) degrading bacteria was isolated and identified as Gram-negative, non-motile rod-shaped bacteria. Based on the results from the phylogenetic tree using 1372 bp sequence of 16S rRNA gene and pairwise similarity results using

GenBank database, the strain was identified as *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T). The 16S rDNA analysis placed strain C_MAR_22_212 in a clade with the species *Klebsiella pneumoniae* subsp. *pneumoniae* and revealed pairwise similarities ranging from 100 to 99%. *K. pneumoniae* occurs among varied fresh and salt water surroundings, soil and plants [60]. *K. pneumoniae* is an opportunistic Gram-negative bacterial enteric pathogen which occurs as hospital acquired infections (HA) and neonatal asepsis [61,62]. *K. pneumoniae* has the potential to produce capsular polysaccharides and is also extensively studied for the bioremediation of heavy metals, like arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu) and nickel (Ni), nitrogenous compounds removal and diclofenac drug removal [63-66]. *Klebsiella pneumoniae* WAH1 has been reported to degrade anti-inflammatory drug diclofenac (10 mg/L) upto 79.14% drug within 72 hr [66]. Bacteria, fungi and algae have been reported for the remediation of acetaminophen contaminated water.

4. CONCLUSION

Pharmaceutical residues have been reported in surface water, drinking water and wastewater treatment plants. Detoxification or removal of such pharma micro pollutants is need of hour to avoid their eco-toxicological effects. Compared to physico-chemical processes, biological method or the combinatorial approach should be used for the elimination of toxic pharma micropollutants or their intermediates. Based on our study, it can be concluded that some pathogenic strain, like *Klebsiella sp.* has potential to degrade paracetamol. But considering the eco-toxicity of its degradative product 4-aminophenol, strain improvement has to be done for the alteration of degradative pathway to yield less eco-toxic degradative metabolite.

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