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ORIGINAL ARTICLE **OPEN ACCESS**

Bioremediation of Acetaminophen and Hydroxychloroquine by biosurfactant producing strain *Bacillus velezensis*

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ABSTRACT

Water-soluble medications that are present in the environment have negative effects on species that are not their intended targets. The pharmaceuticals acetaminophen (paracetamol, APAP) and hydroxychloroquine are emergent micro pollutants during and after Covid. To reduce the influence of pharmaceutical micropollutants on the ecosystem, it is preferable to remove them from the environment with the aid of microorganisms. In this investigation, microorganisms from industrial effluent that could withstand acetaminophen and hydroxychloroquine were identified. Biochemical analysis and the 16s rRNA sequencing method were used to identify the isolate as Bacillus velezensis (GenBank accession number OQ932827.1). A combination of spectrophotometric and high-performance liquid chromatographic methods was used to study the biodegradation of acetaminophen and hydroxychloroquine. The degradation of paracetamol (1500 ppm) and hydroxychloroquine (200 ppm) by Bacillus velezensis was 80.2% and 40%, respectively with simple first order degradation kinetic. Degradative products were reported based on the High-resolution mass spectroscopy data. Based on high resolution mass spectroscopy data, hydroquinone and maleic acid were identified as the acetaminophen's degradative components. The biodegradative metabolite of the parent chemical, 7 Chloroquinoline 4 amine, is suggested by the HRMS chromatogram data for the hydroxychloroquine biodegradative product. Due to their affordability and environmental friendliness, biologically based methods (industrial, hospital, and municipal) for the removal of medicines from wastewater are ideal.

KEYWORDS: Acetaminophen, Biodegradation, *Bacillus velezensis*, Covid ,Hydroxychloroquine,

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INTRODUCTION

Pharmaceutical drugs usage has been increased with the increase in the population and diseases. Pharmaceutical micropollutants are leading to the development of antibiotic and antiviral drug resistance and the possibility of transfer of this resistance genes among other microorganisms. During covid 19 pandemic situation, for the treatment of patients and post vaccine, antiviral and painkiller drugs were generally used [1,2].

Hydroxychloroquine persists in the environment due to its bio accumulative properties in vegetation through the polluted soil and groundwater [3,4,5]. Even natural degradative products of hydroxychloroquine have more toxicity due to their bio resistance [6].

Paracetamol (Acetaminophen; N-acetyl-para-aminophenol) is one of the widely used analgesic and antipyretic drug which is emerging as pharma pollutant worldwide [7]. There are reports on degradation of paracetamol using physico-chemical process and biological process (aerobic and anaerobic degradation, membrane bioreactor and phytoremediation) [8]. Biodegradation of pharma micropollutants like paracetamol has been gained interest among researchers due to low cost and efficacy. Bacteria viz., *Rhodococcus ruber, Stenotrophomonas, Pseudomonas, Delftia tsuruhatensis, Cunninghamella echinulate, Shinella* has paracetamol biodegradative potential [9-13]. As compared to the biodegradation of acetaminophen, biodegradation of hydroxychloroquine is understudied topic. Microbial cell factories have been proved to be efficient for the remediation of such xenobiotic compounds [14].

Acetaminophen and Hydroxychloroquine are water soluble drugs with reported ecotoxic effects on native inhabitants of water bodies. Various Physico chemical processes are available for the removal of pharma pollutants from the pharma industry wastewater and hospital wastewater. High cost, need of space and

generation of secondary pollution are the main critical factors which can be resolved with the help of microorganisms or microbial metabolites like enzymes, surfactants, bio flocculants etc.

MATERIAL AND METHODS

Screening, isolation, and identification of Acetaminophen (APAP) and hydroxychloroquine (HCQ) degrading Bacteria

Pharma Industrial waste water sample was used for the isolation of drugs degrading bacteria. In separate experiment minimal salt medium containing APAP (100 -2500 ppm) and HCQ (50-500 ppm) concentration as carbon source was used for the isolation of microorganisms with the potential to tolerate drug. Bacterial isolates were maintained on nutrient agar plates. Lethal concentration (LC 50) value was determined for both drugs as per the method of Palma et al., 2021 [15]. The isolated bacteria were classified using the data of phenotypic and genotypic characteristics. Preliminary characterizations have been grounded on the morphological, and biochemical tests (Sugar (Mannitol, Glucose, Xylose, Lactose) fermentation tests, Amylase, Gelatinase and Nitrate reduction tests). Identification of the isolate was done using 16s rRNA sequencing method for the confirmation.

Screening of bacteria for laccase, amidase, and surfactant production

Isolate was screened for laccase and amidase enzyme using plate screening method [16- 17]. Strain was screened for the biosurfactant potential using hemolysis assay [18], microplate assay [19], and drop collapse assay [20]. Surface tension reduction in fermented LB and MS broth spiked with APAP and HCQ, was assessed using Stalagmometric Method [21].

Biodegradation Studies

Isolate was inoculated in Bushnell Haas broth (PH 7) containing APAP in the concentration of 1500 ppm and HCQ in the concentration of 200 ppm, in separate batches. For acetaminophen degradation studies, incubation of the flask was done at room temperature in dark condition for 4 days with intermittent determination of cell concentration (OD 600nm), PH and residual concentration of APAP using spectrophotometric based method. BH medium containing HCQ (200 ppm) was incubated at room temperature for 12 days with intermittent checking of cell concentration, pH and HCQ. Residual concentration of hydroxychloroquine was determined by taking absorbance of cell free supernatant at 343 nm.

The degradation /reduction percentage of APAP and HCQ was calculated by Eq. 1:

$$\text{Rate of degradation} = (C0-Ct)/C0 \times 100 \quad (1)$$

C0 = initial concentration of drug; Ct = concentration of drug after incubation at time 't'.

For the characterization of degradative metabolite of drug acetaminophen and hydroxychloroquine, bacterial filtrate was concentrated at 50^0C till dry residue. Residue was further processed for high performance liquid chromatography, for the analysis of degradative metabolite. Qualitative HPLC was performed as per generic acidic method. Waters Alliance 2695 HPLC with PDA detector 2996 instrument was used. Two solvent systems were used consisting of Mobile phase A -Water: Acetonitrile: Fumaric acid (95:05:0.1) and Mobile phase B -Water: Acetonitrile: Fumaric acid (10:90:08). Gradient elution pattern was used with 1 ml/min flow rate. Empower2 software was used for the data analysis.

Identification of degradative metabolites by TLC and HR MS studies

Thin layer chromatography and HR MS methodology was implemented for the characterization of degradative metabolite/s. TLC of acetaminophen and its biodegradative metabolite was performed using solvent system Ethyl acetate: Petroleum ether (1:1) and UV 365nm developer. TLC of hydroxychloroquine and its biodegradative metabolite was performed using solvent system Dimethylamine: Toluene: Isopropanol (1:4:5) and UV 365nm developer.

The mass of the standard and its biodegradable metabolite isolated from fermented broth were analysed using a Bruker Impact HD instrument. Full scan mass spectra from 50 to 1200 m/z were acquired with a scan rate of 4.0 spectra/s using a dual electrospray ionisation source operating in positive ion mode. The source gas was configured to flow at 7.01 l/min and a temperature of 200°C. The injection volume for the sample was 10 µL. The pressure in the nebulizer was changed to 1.7 bar. The software Bruker Compass Data Analysis 4.2 was used to do the data analysis.

RESULTS AND DISCUSSION

Screening, isolation, and identification of Acetaminophen (APAP) and hydroxychloroquine (HCQ) degrading Bacteria

Enrichment of acetaminophen degraders was done using Bushnell Haas Medium containing acetaminophen (100-3000mg/L) concentration. Bacterial Culture showing maximum tolerance to

acetaminophen were sub cultured on nutrient agar plates. Total 23 isolates were obtained but isolates showing high APAP tolerance were further studied.

The isolate showing tolerance to 2000 ppm concentration of APAP and 200 ppm concentration of hydroxychloroquine, was found to be Gram-positive rod-shaped bacteria with cream-colored colonies . Based on the biochemical tests, isolate found to be catalase, amylase, gelatinase positive, glucose fermentative, methyl red positive and non-spore forming. Molecular identification was done by 16S rDNA sequencing method. Isolate was identified as *Bacillus velenzies* with 99.65% similarity and 100 % query cover. The sequence of the strain was deposited in GenBank database with OQ932827.1 accession number [22]. Phylogenetic dendrogram (Fig.1) was plotted using neighbor joining method. The strain belongs to Bacillota phylum, Bacilli class, Bacillales order, family Bacillaceae and Bacillus genus.

Bacillus velezensis is Gram positive, non-spore forming bacteria. This strain has been previously reported in the remediation of tetracycline and antihypertensive drug Prazosin (PRZ)[23].

Screening of bacteria for laccase, amidase, and surfactant production

The strain was found to have the potential of laccase and biosurfactant production but it is amidase negative (Fig 2-5 and Table 1).

Biodegradation studies

Based on the spectrophotometric method, *Bacillus velezensis* showed 80.2% and 40 % degradation of paracetamol (1500 ppm) and Hydroxychloroquine (200 ppm) after 4 and 12 days of incubation respectively. In stationary phase, bacteria showed the more degradative potential. Changes in pH was observed during acetaminophen degradation where pH changes from 7 to 7.36 . Hydroxychloroquine biodegradation showed shifts towards acidic environment (7 to 5.6). Time course effect for acetaminophen and hydroxychloroquine biodegradation is given in Fig.6. Kinetic parameters involved during biodegradation are given in Table 2.

Qualitative HPLC was carried using generic acidic method. Based on the acquired HPLC chromatographic data, it can be inferred that the strain has the potential to degrade acetaminophen (Fig 7 ,8) and hydroxychloroquine (Fig.9,10). For the identification of biodegradative metabolite of acetaminophen and HCQ, TLC and HR MS techniques were used.

Identification of degradative metabolites by TLC and HR MS studies

Acetaminophen and hydroxychloroquine biodegradative metabolites were analyzed using TLC and high resolution mass spectroscopic studies. Chromatography of standard hydroquinone, acetaminophen and biodegradative metabolite, suggested that *Bacillus velenzesis* could degrade acetaminophen into hydroquinone (Fig.12). As per the TLC data, HCQ was degraded into metabolites having Rf value of 0.67 and 0.33 (Fig.11). For further confirmation of degradative metabolites, HRMS studies was conducted which suggests formation of hydroquinone (Mol.Wt 108) and maleic acid (mol wt 116) due to microbial action on acetaminophen (Fig.13). Whereas 7 chloroquine 4 amine was identified as degradative product from HCQ (Fig.14).

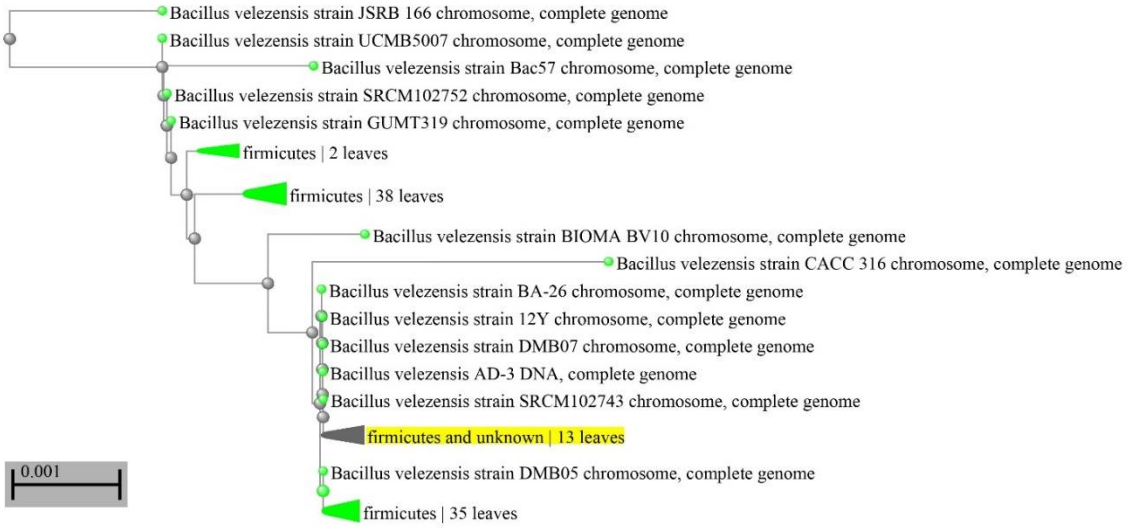

Fig.1 Phylogenetic tree of the strain

Fig.2 Laccase positive *Bacillus velezensis*

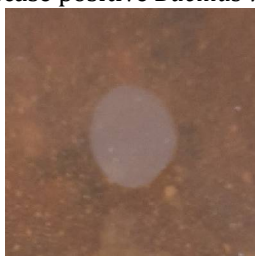

Fig.3 Amidase negative *Bacillus velezensis*

Fig.4 Hemolysis assay using *Bacillus velezensis*

Fig.5 Foam production(due to biosurfactant) in Inoculated broth of BH medium spiked with HCQ

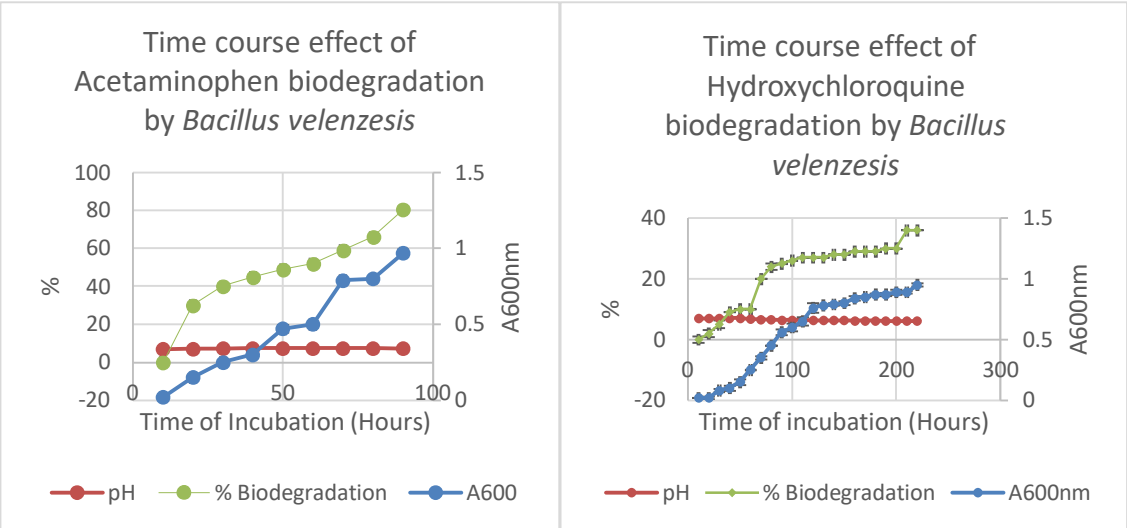

Fig.6 Time course effect of Acetaminophen and Hydroxychloroquine biodegradation by *Bacillus velezensis*

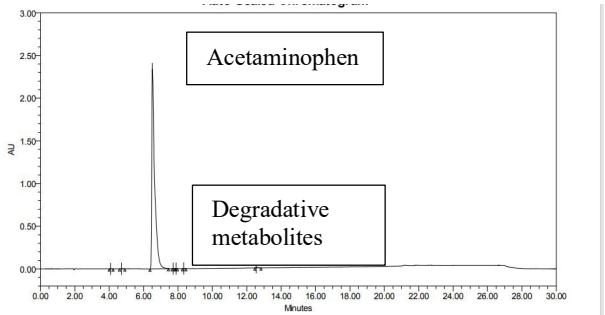

Fig. 7 Reverse phase HPLC Chromatogram of extracted metabolite from *Bacillus velenzesis* fermented broth containing 1500 ppm of Acetaminophen

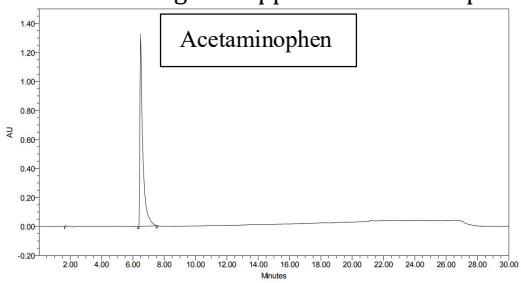

Fig. 8 Reverse phase HPLC Chromatogram of standard acetaminophen

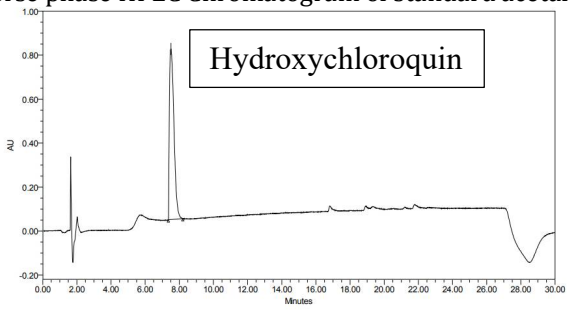

Fig. 9 Reverse phase HPLC Chromatogram of extracted metabolite from *Bacillus velenzesis* fermented broth containing 200 ppm of Hydroxychloroquine (HCQ)

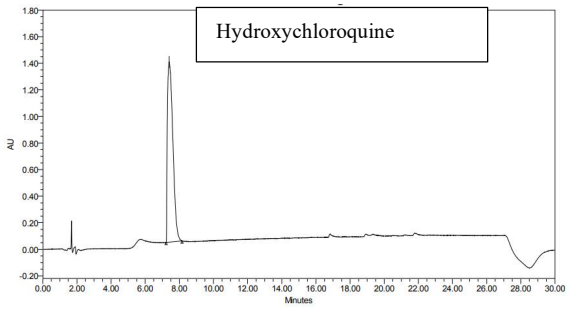

Fig.10 Reverse phase HPLC Chromatogram of Hydroxychloroquine (HCQ)

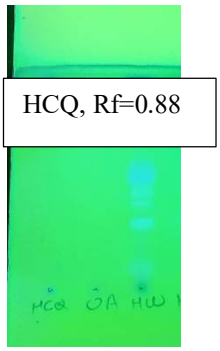

Fig.11 TLC of Hydroxychloroquine and biodegradative metabolites using Dimethylamine: Toluene: Isopropanol (1:4:5) And UV 365 nm as developer.

Fig.12 TLC of Acetaminophen and biodegradative metabolites using Ethyl acetate: Petroleum ether (1:1) and UV 365nm developer.

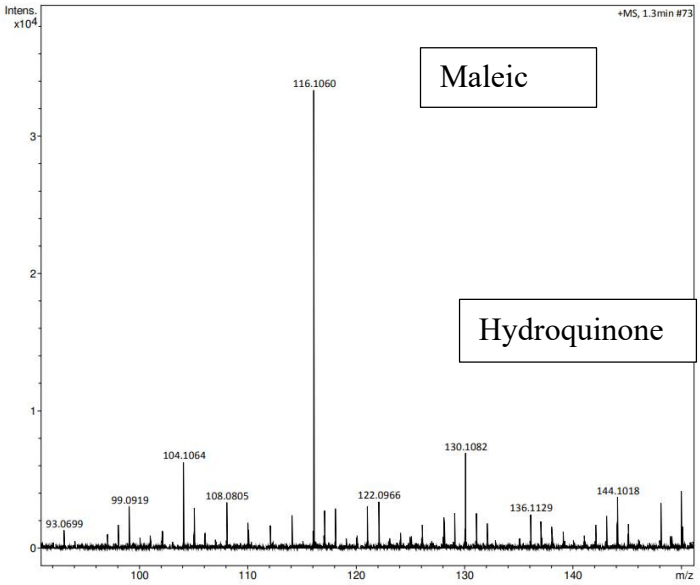

Fig. 13 HRMS of acetaminophen biodegradative metabolites Hydroquinone and Maleic acid

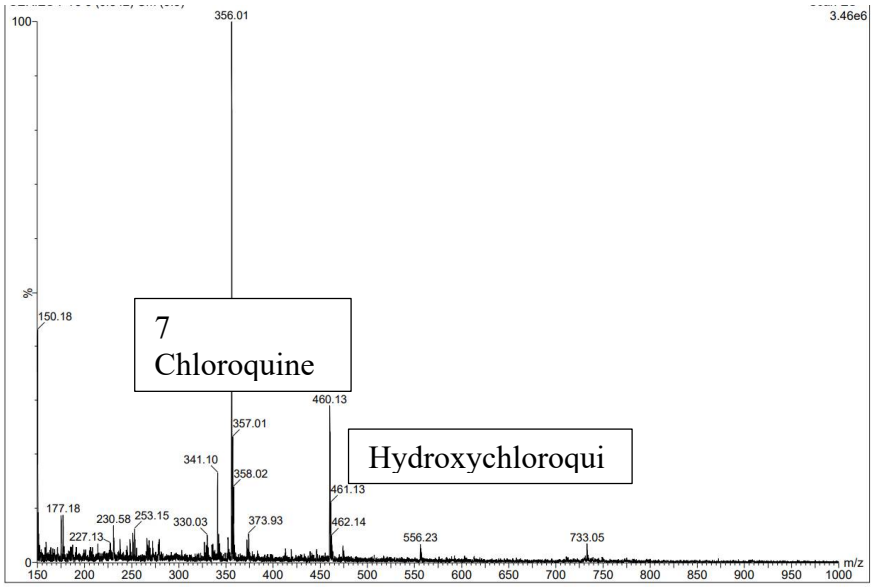

Fig. 14 HRMS of hydroxychloroquine biodegradative metabolite 7 Chloroquine 4 amine

Table.1 Screening of *Bacillus velezensis* for biosurfactant production

Name of the method	Inference
Hemolysis assay	Positive
Drop collapse assay	Positive
Microplate assay	Positive
Surface tension reduction using Stalagmometric Method	
Luria broth	59 %
Mineral salt broth with APAP	38.42 %
Mineral salt broth with HCQ	40.73 %

Table. 2 Acetaminophen and Hydroxychloroquine biodegradation kinetic model and parameters

Biodegradation of-	Kinetic Model	Parameter	Chi-sq error	50 % Degradation Time (days)	90% Degradation Time (days)
Acetaminophen	Simple first order	k: 0.268	11.7	2.58	8.58
Hydroxychloroquine	Simple first order	k: 0.0626	13.4	11.1	36.8

Among the genera of *Bacillus* group, *Bacillus subtilis* subsp. *subtilis* NCIB 3610(T) has been reported to degrade 73.2 % of acetaminophen (2500 ppm) into 4- aminophenol [24]. *Bacillus drentensis* strain S1 showed the potential to degrade 300 ppm of acetaminophen into hydroquinone and oxalic acid along with -isopropyl-5-methylcyclohexanone and phenothiazine as intermediates [7]. *Bacillus cereus* have been reported to degrade acetaminophen (200 ppm) after 144 h of incubation into hydroquinone, 4-aminophenol, and 2-hexenoic acid [15].

Photo mediated and advanced oxidation process mediated degradation of hydroxychloroquine generates 7 Chloroquine 4 Amine and Oxalic acid [25]. In our study, we could find out 7 chloroquine 4 amine as product of HCQ (Fig.14).

CONCLUSIONS

In summary, a *Bacillus* strain that can degrade 80.2% and 40% of acetaminophen and hydroxychloroquine was isolated and identified as *Bacillus velezensis*. More importantly, to the best of our knowledge, this is the first study to demonstrate biodegradation of hydroxychloroquine. It showed that the degradation capability was attributed to the potential of laccase enzyme production. Therefore, the laccase enzyme

produced by the strain is the promising agent for the removal of drugs from the polluted site. However, further research such as strain improvement program, and optimization of fermentation conditions are needed to enhance the biodegradation efficiency for hydroxychloroquine.
Meanwhile, more research is needed to identify drug-isolate interactions, and elucidate the mechanisms of drug uptake or degradation, to provide new insights into the pharma micropollutants bioremediation.

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Conflict of Interest: Nil

Author's Contribution: Manuscript preparation and work has been done by Meghmala Waghmode, under the guidance of Prof. Neha Patil.

Funding: Nil

Ethics Statement: NA

Informed Consent: NA

Data Availability: The datasets generated during and/or analysed during the current study are available from the corresponding author (Meghmala Waghmode, meghmicro@gmail.com) on reasonable request.

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