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First Report of *Enterobacter hormaechei* Isolated from Agricultural Soil in the Biodegradation of Glyphosate

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ABSTRACT

Several studies have explored the utilization of soil microorganisms, to address the environmental issues associated with glyphosate use and enhance crop yields. In our investigation, screening on Agar plate and broth medium Luria Bertani was carried out after isolating bacterial strains from rhizospheric agricultural soil in Mascara, Algeria, to biodegrade glyphosate, following that by testing the Plant Growth-Promoting Rhizobacteria and evaluate the effects of glyphosate on these proprieties. Our findings indicate that five bacterial strains exhibited growth in the presence of glyphosate concentrations up to 25 mg/ml, beyond this concentration the strains have developed tolerance. Following a partial examination of the 16S rRNA sequences, the bacterial strains were identified as belonging to the genus of *Enterobacter*. After 10 days of incubation with the glyphosate, Phosphate solubilization decreased in broth and agar Pikovskaya medium and the bacterial strains synthetized less of indole-3-acetic acid compared to the control, indicating the impact of glyphosate on these outcomes, high concentration of glyphosate inhibited nitrogen fixation, and various doses of glyphosate were found to restrict the growth of biofilms in these strains. The results of HPLC examination of secondary metabolites revealed that the primary degradation products of glyphosate in all strains were Sarcosine and Glycine. So, it seemed that the strain could both biodegrade glyphosate and use it for growth ,while also possessing rhizobacteria properties that promote plant development, enabling the use of the strains in the bioremediation of glyphosate-contaminated soils.

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INTRODUCTION

Glyphosate is the active ingredient of phosphonate herbicides (Singh & Singh, 2016). It is an acid with a stable C-P bond, which is frequently used as a salt of isopropylamine. It is a non-selective, post-emergence, non-systemic, competitive and broad-spectrum herbicide, which makes glyphosate the most widely used herbicide in the world. It is used to kill annual and perennial plants (Chen et al., 2022; Duke, 2020; Hove-Jensen et al., 2014; Gill et al., 2018; Wijekoon & Yapa, 2018; Xu et al., 2019). This reveals its herbicidal activity by the inhibition of EPSPS, key enzyme used in the synthesis of the essential aromatic amino acid, resulting in the inhibition of secondary metabolites in plants. This enzyme is present in plants and microorganisms but not in human or animal cells (Mertens et al., 2018; Zhan et al., 2018). Glyphosate has been shown to have genotoxic effects and to affect the activity and expression of estrogen transcription receptors (ERs). Additionally, its inhalation by humans can cause DNA damage, it has also shown human health risks such as DNA damage caused by human

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inhalation, cardiotoxicity, carcinogenicity, skin toxicity, endocrine disruption, and reproductive disorders. In 2015, the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) classified glyphosate as possibly carcinogenic (category 2A) after epidemiological, animal and in vitro studies (Hernández-Alomia et al., 2022; Koller et al., 2012; Thongparakaisang et al., 2013). The removal of glyphosate from the environment is necessary to eliminate induced toxicity. For this purpose, Glyphosate can be degraded through both abiotic methods, such as adsorption and photolysis (Manogaran et al., 2017), and biotic degradation by microorganisms species who used glyphosate as a source of Carbone or Phosphorus-utilizing bacteria such as *Flavobacteria*, *Acetobacter*, *Arthrobacter*, *Azotobacter*, *Pseudomonas*, *Alcaligenes*, *Bacillus*, *Enterobacter* and *Klebsiella* have been implicated in the biodegradation of glyphosate (Chennappa et al., 2014; Wijekoon & Yapa, 2018; Xu et al., 2019). There are two possible biodegradation pathways associated with glyphosate degradation. The first pathway involves the cleavage of the carbon-nitrogen (C-N) bond by the enzyme glyphosate oxidoreductase, leading to the production of aminomethylphosphonic acid (AMPA). AMPA can further be metabolized to methylamine and phosphate by the enzyme C-P lyase (Fan et al., 2012; Singh et al., 2019). Several microorganisms have been identified to carry out this pathway, including *Agrobacterium radiobacter* SW9, *Arthrobacter atrocyaneus* ATCC 13752, *Flavobacterium sp*.GD1, *Geobacillus caldoxylosilyticus* T20, *Ochrobactrum sp*. GDOS, *Pseudomonas sp*. LBr, *Aspergillus oryzae* A-F02, *Penicillium chrysogenum*, and *Trichoderma harzianum* (McAuliffe et al., 1990; Pipke & Amrhein, 1988; Balthazor & Hallas, 1986; Obojska et al., 2002; Hadi et al., 2013; Jacob et al., 1988; Fu et al., 2017; Klimek et al., 2001; Krzyśko-Lupicka et al., 1997). The second pathway involves the cleavage of the carbon-phosphorus (C-P) bond by the enzyme C-P lyase, leading to the formation of Sarcosine is then oxidized into glycine and formaldehyde by the enzyme sarcosine oxidoreductase (Zhan et al., 2018). Microorganisms such as *Alcaligenes sp*. GL, *Streptomycete sp*. StC, *Enterobacter cloacae* K7, *Achromobacter* sp. strain MPK 7A, and *Achromobacter sp*. MPS have been found to utilize this pathway for glyphosate degradation (Lerbs et al., 1990; Obojska et al., 1999; Kryuchkova et al., 2014; Ermakova et al., 2017; Sviridov et al., 2012). Interestingly, *Comamonas odontotermitis* P2 and *Bacillus cereus* CB4 have been shown to degrade glyphosate through both the AMPA and sarcosine pathways (Fan et al., 2012; Firdous et al., 2017). The rate of glyphosate degradation by these microorganisms is influenced by environmental conditions. Numerous studies have demonstrated that microorganisms belonging to the Plant Growth-Promoting Rhizobacteria (PGPR) group exert beneficial effects on plant health through various mechanisms, including nutrient mineralization, disease suppression, increasing plants resistance to natural stress, synthesizing growth-promoting substances such as production of phytohormones (acid indole-3-acetic),N_2 fixation , solubilization of insoluble phosphorus, production of siderophores, among which we have *Azospirillum*, *Pseudomonas*, *Klebsiella*, *Azotobacter*, *Enterobacter*, *Alcaligenes*, *Bacillus*, *Burkholderia* and *Serratia*. However, these PGPRs have the capacity to use glyphosate by synthesizing a naturally appropriate enzymes (Hadi et al., 2013; Kryuchkova et al., 2014; Kumar et al., 2017; Shahida et al., 2019; Sezen et al., 2016; Travaglia et al., 2015; Wijekoon & Yapa, 2018). The present invistigation has two objectives the first aim is to isolate and characterize bacterial strains with the ability to degrade glyphosate analysis the pathway of glyphosate degradation products. The second aim is to screen of the selected strains for various PGP properties such as biofilm formation, nitrogen fixation, phosphorus solubilization and indole-3-acetic acid production (IAA) and to evaluate the impact of glyphosate on PGPR traits

MATERIALS AND METHODS

Soil sampling

In this study, soil samples were taken from a cereal-growing agricultural soil in Mascara,

a town in western Algeria. The samples were taken from depths of 0-30 cm at five different points in the same field. We collected 100-150 g of soil, homogenized it, and stored it in sterile bags at 4 °C. One part was used to isolate bacterial strains and the other part was used for physicochemical analyses of soil characteristics.(Ermakova et al., 2010; Kaczynski et al., 2020; Xu et al., 2019)

Isolation and purification of glyphosate degrading bacteria

The isolation was initiated by suspending the samples in sterile saline solution (1 g.100 mL^{-1}) under vortexing for 90 minutes at 30°C. The purification was performed on Luria Bertani (LB) agar medium. Pure soil bacterial strains were then grown in LB agar medium supplemented with 6.12 mg/ml N-(phosphonomethyl) glycine (Tiller48%, purchased from a local supplier of agricultural products in Algiers, Algeria). The cultures were incubated in triplicate at 30°C for 24-72 hours. Then the selected isolates were placed on LB medium with 12.5 mg/ml of glyphosate in triplicate and incubated at 30°C for 24-72 hours. Additionally, a subset of isolates was incubated at a higher concentration of glyphosate (200 mg/ml) to ensure the ability of isolated bacteria to grow and degrade glyphosate (Elarabi et al., 2020).

Screening of glyphosate degradation bacteria in broth medium

Afterward , the selection of the best-performing isolate was analysed in the presence and absence of glyphosate. The test was carried out in Elisa plates, (140 µl) of nutrient broth (NB), supplemented with glyphosate (40 µl) at (6.12, 12.5, 25, 50, 100, 150, 200 mg/ml).. The medium were inoculated with (10 µl) of each isolate from cultures with an optical density of A= 0.6 at 630nm. The isolates were also grown in NB without the presence of the herbicide. Plates were incubated at 30°C for 24h-48hours and absorbance was measured with an BioTek ELx808 Microplate Reader at 630nm (Melo et al., 2016).

Strain identification

The isolated microorganisms were identified by all phenotypic methods, such as morphological and biochemical reaction test Gram's reaction, Catalase, Oxidase test, Nitrate reductase, Urease, Voges-Proskawaur, Citrate and indole production (Benslama & Boulahrouf , 2013; Bhatt & Iyer, 2020; Kryuchkova et al., 2014) and by Genotypic methods, based on partial 16S rDNA gene sequencing. Bacteria were grown in NB agar for 48h at 37°C, The genomic DNA of each culture was extracted using Nucleospin of Macherey-Nagel Kit (Germany). The partial sequencing of the 16S rDNA gene of the bacteria was amplified by polymerase chain reaction (PCR) under the following conditions: preheating (94°C, 5 min), followed by 35 cycles of denaturation (94°C, 30 s), hybridization (55°C, 45 s) and amplification. (55°C, 45 s) and extension (72°C, 40 s) and finally extension (72°C, 5 min); using the direct primer 27f (5' AGAGTTTGATCCTGGCTCAG 3') and the reverse primer 1492R (5' GGTTACCTTGTTACGACTT 3') (Heuer et al., 1997). The purified PCR product was then sequenced according to Sanger et al., 1977, using the Big Dye Terminator Cycle Sequencing Kit V3. 1(Applied Biosystems, USA) following the manufacturer's protocol. Phylogenetic tree was constructed using the MEGA (Molecular Evolutionary Genetics Analysis) software version 6

Effect of environmental conditions on bacterial growth

Many parameters can affect the bacterial isolates growth such as temperature, pH, salt concentration, nutrient availability, presence of pesticides etc. Among these,all bacterial isolates were inoculated and incubated at different temperatures (25°C, 37°C and 44°C) , with different pH ranges(4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) and different NaCl concentrations (1.0, 2.0, 3.0 et 4.0 %) in nutrient broth for 18 hours and 24hours with (12.5 mg/ml) of glyphosate. The absorbance of the bacterial isolates from the nutrient broth was measured at 600 nm (Malviya et al., 2015).

Growth Kinetics

To determine isolate growth after glyphosate addition, the isolates strains were inoculated in nutrient broth supplemented with two different concentrations of glyphosate 12.5 and 25 mg/ml (all strains were growing 24hours in nutrient broth) and incubated at 37°C for 120 hours in shaking conditions (120 rpm). The NB containing isolates without glyphosate was used as the control. The growth kinetics was followed by monitoring the optical density of the medium for 120 hours using a UV/VIS spectrophotometer at 620 nm (Malviya et al., 2015; Parakhia et al., 2019).

Plant Growth-Promoting Rhizobacteria activity assays and their evaluation under herbicide stress

The Phosphate solubilization

The bacterial strains were tested under in vitro conditions in the presence and absence of glyphosate for P-solubilization. The bacterial strains were inoculated into a Pikovskaya agar medium without glyphosate and another Pikovskaya medium agar supplemented with 12.5 mg/ml of glyphosate, and incubated at 37°C for 7 days and the halo formation around the bacterial colony was observed and measured. The strains were also used to determine the amount of P-solubilisation in which the bacterial colonies was inoculated with 1 ml of bacterial culture (10^8 cells mL^{-1}) and incubated at 37 °C with agitation (at 120 rpm) in Pikovskaya broth completed with 12.5 mg/mL of glyphosate. Available phosphorus was measured in the bacterial supernatant 5, 8 and 10 days after incubation. The amount of P-solubilization was calculated using the KH2PO4 calibration curve (Ahemad & Khan, 2012; Shahid et al., 2021).

Quantitative assay of acid indole-3-acetic (IAA)

The acid indole-3-acetic (IAA) synthetized by the rhizobacterial strains was analysed quantitatively. For this assay, 1 ml of the rhizobial strains (10^8 cells/ml) was inoculated into LB broth (100 ml) containing tryptophan (100 µg ml^{-1}) and supplemented with 12.5mg/ml glyphosate after incubation with agitation at 125 rpm for 7 days at 28 ± 2°C. The inoculated broth without glyphosate was used as a control (Chennappaet al., 2014). 5 ml of each culture was centrifuged (8000g) for 10 min and 2 ml of the supernatant was mixed with 100 ml orthophosphoric acid and 4 ml Salkowsky's reagent , the reaction was incubated at 30°C in the dark for 1 hour. Absorbance was read at 530 nm. The concentration of IAA in the supernatant was determined using a calibration curve of pure IAA as a standard (Ahemad & Khan, 2012; Chennappa et al., 2014; Shahid & Khan, 2018).

Biofilm formation

This test was performed by inoculating 5ml of the bacterial culture incubated at 30ºC for 48 hours in test tubes, supplemented with glyphosate (6.12,12. 5,25,50,100,150,200 mg/ml). 250 µl of this culture was transferred to a microtiter plate and incubated for 24hrs, 48hrs at 30ºC after this incubation the culture was gently discarded and tapped onto a filter paper, then 250 µl of 0.1% crystal violet was added to the bacterial strain wells and incubated at room temperature for 30 min. The crystal violet was removed by washing it with distilled water. Finally, the absorbed crystal violet was extracted with 250 µl of 95% ethyl alcohol for 1 hours; absorbance was taken at 630 nm (Ahemad & Khan, 2012; Kumar et al., 2017).

Nitrogen fixation

Atmospheric nitrogen fixation was determined using Ashby's N medium, based on the growth of the rhizospheric strains.The bacterial strains were inoculated into Ashby's N medium supplemented with glyphosate (12.5 mg/ml) and incubated for 5 days at 37°C, while Ashby's N medium without glyphosate was used as a control (Kryuchkova et al., 2014).

Detection of degradation residues by HPLC

To determine the biodegradation of glyphosate metabolite, bacterial strains were grown in NB with 12.5 mg/ml of glyphosate under agitation at 180 rpm for 5 days. The culture were then centrifuged at 8000 rpm for 15 minutes, washed twice with phosphate buffer (0.05 M; pH 7.0), resuspended in the same buffer and disintegrated using an ultrasound probe (45 cycles). The homogenate was centrifuged at 10000 rpm for 15 min and the supernatant was collected (Fan et al., 2012; Zhao et al., 2015). 0.12 mL of 5% borate buffer and 0.12 mL of 12,000 $mg.L^{-1}$(p-toluenesulfonyl) acetonitrile was adding to 1 mL of aqueous sample at pH 9.0. The reaction was carried out at 22 °C for approximately 16 hours, the reaction was then stopped and the derived samples were filtered through 0.45 mm syringe filters and 10 µL was injected into the chromatograph using a C18 column with a HAc/NH4Ac (pH 4.8)-acetonitrile gradient of 5 $mmol.L^{-1}$. The separated components (glyphosate, AMPA, sarcosine and glycine was detected at 290 nm. Standards were prepared using the same protocol(Xu et al., 2019).

Statistical analysis

The treatment of all experimental data was analysed by Shapiro-Wilk's normality,Friedmen test(R Core Team, 2022) ($W = 0.91692$, $p < 0.001$). Therefore, in the study of Phosphate-solubilization, the two way ANOVA and the Post hoc test (Honesty Signicance Difference de Tukey) was applied using agricolae package ($p<0.05$) (Mendiburu, 2020).

RESULTS AND DISCUSSION

Isolation, screening and identification of the glyphosate-degrading strain

The results showed that five morphologically distinct bacterial strains were isolated from uncontaminated cereal rhizospheric soil and they were able to grow in the presence of glyphosate up to a concentration of 100 mg/ml on LB agar plates, furthermore, an increase in absorbance up to 50 mg/ml of glyphosate was observed in the nutrient broth and the MSM. The obtained rhizospheric bacterial strains with the ability to degrade glyphosate were characterised by morphological analysis and appeared as large, white, smooth, and semi-circular colonies under microscopic observation, the strains seem as Gram-negative short rods. Biochemical identification revealed a positive reaction to catalase, nitrate reduction, Voges' proskaur, citrate and a negative reaction to oxidase, indole reaction and urease. The results of the 16S rRNA sequencing performed confirmed that the strains were *Enterobacter cloacae* and *Enterobacter hormaechei* by comparison with 16S rRNA sequences classified in NCBI. There was a100% similarity between the 16S rRNA gene of CAF-1 and *Enterobacter cloacae* strain F16 (GenBank accession number KX896752.1). The CAF-2 16S rRNA gene was a 99.62% similar to *Enterobacter hormaechei* strain ER46 (GenBank accession No. MT124571.1), a 99.63% similarity was observed between the 16S rRNA sequence of CAF-3 and the sequence of *Enterobacter hormaechei* subsp. xiangfangensis strain 9-PYG-DE-D4-58 with the accession number (MT903205.1), the 16S rRNA sequence of CAF-4 was similar at 99.69% to *Enterobacter hormaechei* strain CEMTC_3874 (GenBank accession number: MW980942.1) The nucleotide sequence of the CAF-5 16S rRNA gene was a 99.63% similar to *Enterobacter hormaechei* strain 8 (C2P2) with the accession number (KF254588.1). The isolated bacterial strains (CAF-1,CAF-2,CAF-3,CAF-4and CAF-5) were characterised and placed in NCBI with the accession number OR031247, OR031248, OR031249, OR031250, OR031251, respectively (Figure 1).

Growth kinetics of the glyphosate-degrading strain

The findings of the current study showed that the bacterial strains were able to grow at a high concentration (25 mg/ml) of glyphosate compared to (12.5mg/ml) concentration; the rhizospheric isolates were able to tolerate concentrations of glyphosate higher than 25 mg/ml.

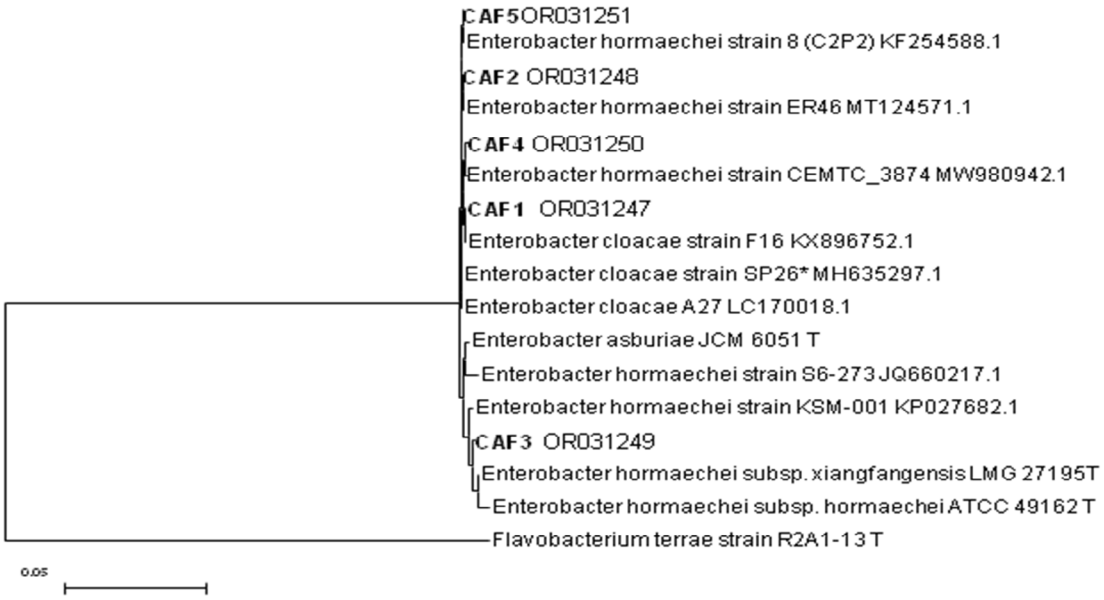

Fig. 1. The Phylogenetic tree was constructed on the basis of partial 16S rRNA gene sequences from selected bacteria and closely related phylogenetic species, obtained using NCBI's BLAS software. The sequences were aligned using MEGA 6.0 software.

After 96 hours of incubation, the strains were growing significantly more than those that were growing in medium containing 12mg/ml of glyphosate. the rate of growth of CAF-2 strain was the fastest at 96 and 120 hours with 25mg.ml-1of glyphosate. Glyphosate may have been used by the isolates as a nutrient source to support their growth (Figure 2). Glyphosate-degrading Enterobacter CAF-1, CAF-2, CAF-3, CAF-4 and CAF-5 showed excellent growth after 96 hours of incubation, suggesting that the strains used glyphosate as a source of nutrients for growth and development (Figure. 2).

Effect of enviromental condition on bacterial growth:

The isolated strains was used to determine the influence of various environmental conditions (PH, Temperature and NaCl concentrations) on the degradation of glyphosate. The growth of the CAF-1, CAF-2, CAF-4 and CAF-5 strains was significant at Ph (7 and 9). In the other hand, the CAF-3 strain was have very significantly growth at all pH levels. Furthermore, our results demonstrated that the growth efficiency was significantly ($p<0.001$) in the neutral and alkaline pH ranges when glyphosate (12.5 mg/ml) was present (Figure 3A) after 18 hours of incubation, The growth evolution of bacteria at 25°C, 37°C, and 44°C in the presence of 12.5 mg/ml of glyphosate revealed that the CAF-2, CAF- 4 and CAF-5 were able to develop at 44°C, but CAF-1 and CAF-3 could only grow at 37°C. With the exception of strain CAF-4, which showed increased absorbance at 44°C, all strains showed good absorbance at 37°C after 24 hours (Figure. 3B). After 18 hours, the optimal concentration of NaCl for strains CAF-1, CAF-2, CAF-3, CAF-4 and CAF-5 in the presence of glyphosate was 4%, 2%, 1% and 3 %, respectively. All strains showed a better absorbance in1% concentration of NaCl after 24 hours under glyphosate stress (Figure. 3C).

In vitro production of plant growth-promoting substances when exposed to glyphosate and in the absence of .

In the current investigation, rhizospheric strains glyphosate degrading were evaluated

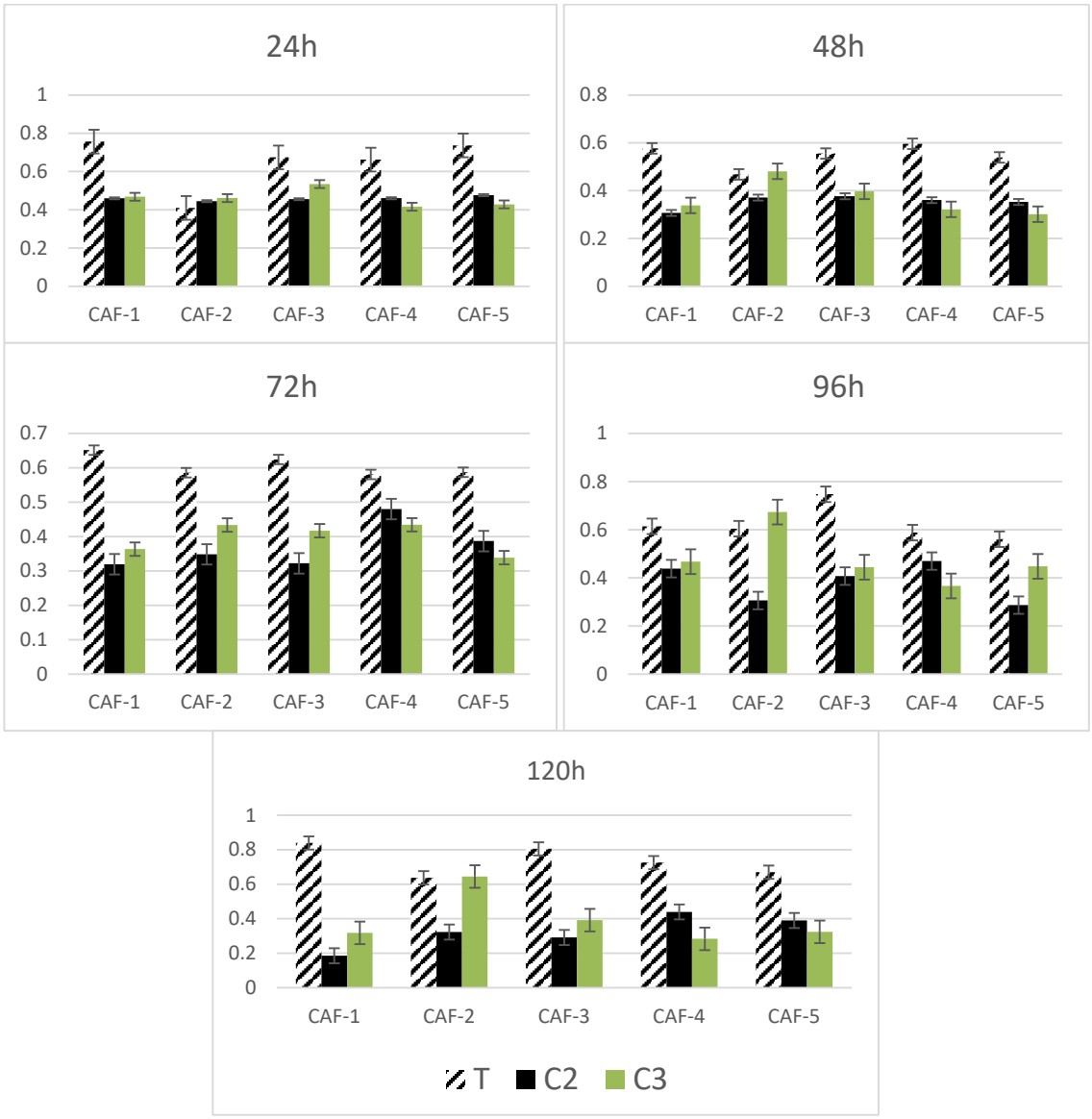

Fig. 2. Growth curve of strain in glyphosate concentration (12.5mg/ml, 25mg/ml) at 37°c for 24h,48h,72h,96h and 120 hours.

for PGPR characteristics such as the solubilization of inorganic phosphorus, production of phytohormones, Nitrogen fixation and biofilm farmation in the presence and absence of glyphosate stress.

Nitrogen fixation

All bacterial strains were found to be able to fix nitrogen on Ashby's N-medium in control dish. As well as on Ashby's N-free medium plates agar containing 12.5 mg/ml of glyphosate the bacterial colonies CAF-1, CAF-2, CAF-3, CAF-4 and CAF-5 were also presented by the growth and change of the colonies colour from white to pink, thus the bacterial strains have the capacity to fix nitrogen at low dose of glyphosate on Ashby's N-medium. (Table 1)

Production of acid indole-3-acetic (IAA)

These results demonstrated how the herbicide affected the production of IAA. IAA production was significantly higher for strain CAF-4 (61.14 ppm ±2.01) compared to CAF-1 (32.73 ±4.69),

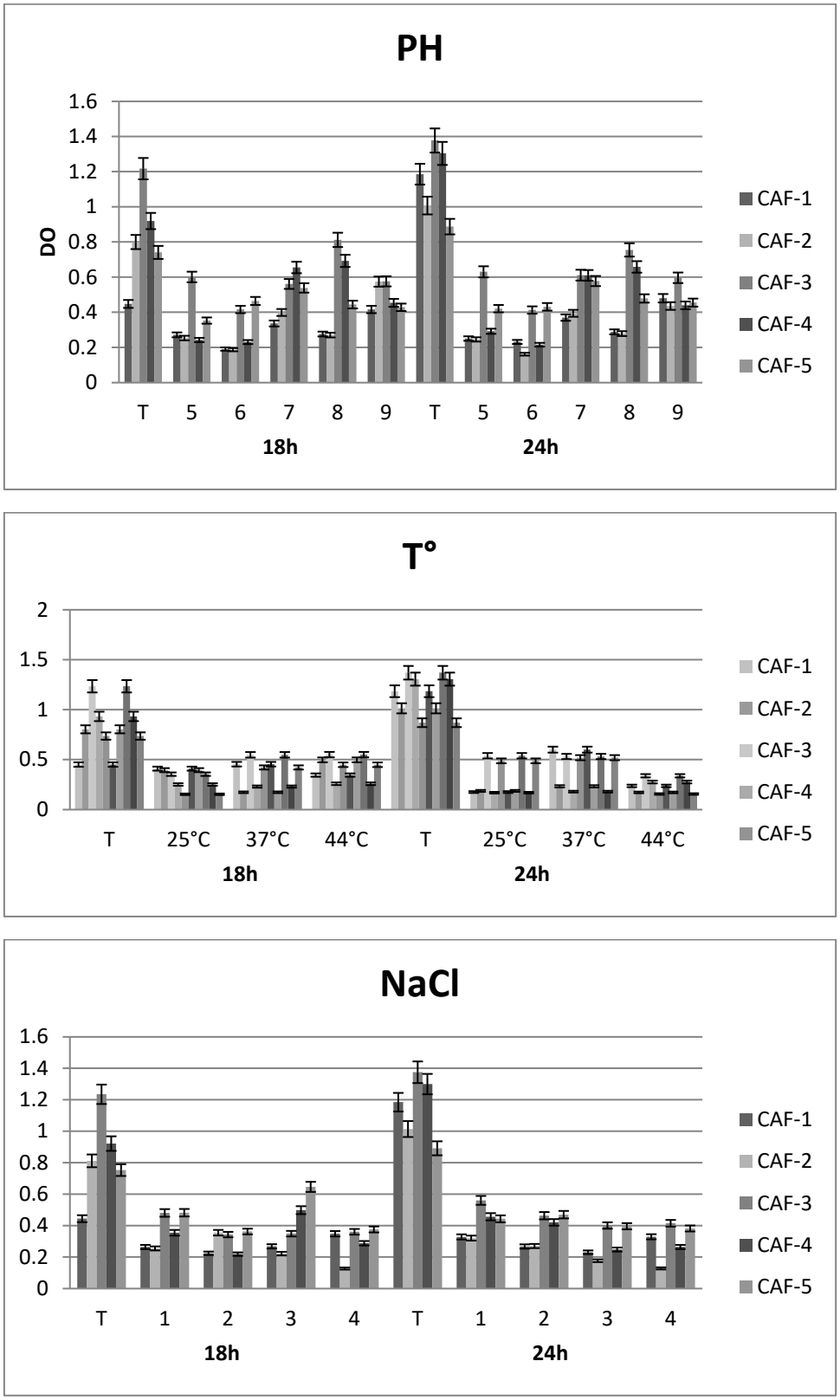

Fig. 3. Effects of pH (a), incubation temperature(b), NaCl concentrations (c)on bacterial growth incubated with 12.5mg/ml of glyphosate concentration

Table 1. Phosphate-Solubilization Activity, IAA production and N fixation of bacterial strains in absence and presence of glyphosate.

	Solubilization zone mm	P solubilization µg/ml			IAA Production	N fixation
souches		day 4	day8	day10		
CAF-1	12	50,37±1,76	60,69±2,81	53,38±2,74	32,73±4,69	+
CAF-2	11	53,93±5,37	73,57±5,08	109,00±4,06	12,30±2,10	+
CAF-3	15	59,14±6,99	56,03±2,70	56,03±5,21	22,07±1,00	+
CAF-4	17	80,87±5,89	82,61±4,21	58,41±3,70	61,45±2,01	+
CAF-5	13	46,17±19,64	31,28±8,49	51,74±2,95	21,05±0,95	+
C2(12.5mg/ml)						
CAF-1	5	1,87±7,75	36,67±1,82	28±10,21	10,84 ±1,30	-
CAF-2	6	21,07±20,01	62,33±6,84	17,58±4,95	9,75±1,08	-
CAF-3	9	1,11±7,43	81,42±2,84	78,50±6,63	4,53±2,46	+
CAF-4	11	40,23±4,60	55,85±3,96	57,13±4,12	13,98±0,68	+
CAF-5	8	3,79±7,95	31,47±1,14	8,54±13,62	4,64±0,89	+

CAF-2 (12.309 ±2.10), CAF-3 (22.078 ±1.00) and CAF-5 (21.05 ±0.95) at the control level ($p \leq 0.001$). Auxin production for CAF-1, CAF-2, CAF-3, CAF-4 and CAF-5 was somewhat decreased after the addition of glyphosate at 12.5 mg/ml compared to control (10.84 ±1.30), (9.75 ±1.08) (4.53 ±2.46), (13.98 ±0.68), and (4.640.89), respectively. The decrease in indole-3-acetic acid synthesis exhibited by a low concentration of IAA was most noted at the highest concentration of glyphosate used. (Table 1)

Phosphate solubilization

Evaluation of the ability of the selected isolates to solubilize phosphate on Pikovskaya agar was observed by the appearance of a clear halo around the colony in the absence and presence of glyphosate. After 7 days incubation all our isolates were able to solubilize P by the formation of halo. The largest being 17 mm formed by the strain CAF-4, which was then significantly reduced to 11 mm in the presence of glyphosate at 12.5 mg/ml (Table 1). Phosphate solubilization on Pikovskaya liquid medium of the control strain CAF-2 increased over time and is highly significant (109µg/ml±4. 06) on day 10 of incubation, the other strains showed significant phosphate solubilization, after addition of glyphosate at 12.5mg/ml. All strains showed a decrease in phosphate solubilization up to 50% compared to the control. However, it was observed that strain CAF-3 has a significant increase in production (81.42±2.84) (78.50±6. 63) respectively compared to the control at 8 and 10 days of incubation as well as strain CAF-5 which shows no change in phosphate solubilization (31, 47±1. 14) at day 8 (Table 1) compared to the control. Thus it can be deduced that some strains can solubilize P in the presence of glyphosate, indicating that glyphosate has no significant effect on phosphate solubilization. (Table 1)

Biofilm formation

Biofilm formation was evaluated using a microtiter plate assay in the current study. Our findings indicate that CAF-1, CAF-2, CAF-3, CAF-4, and CAF-5 strains were capable of forming biofilms. However, exposure to different concentrations of glyphosate inhibited biofilm formation in these isolated strains. This inhibition was observed by the absence of circular formations at the interface of the wells after fixation with methanol. At 24 hours, the presence

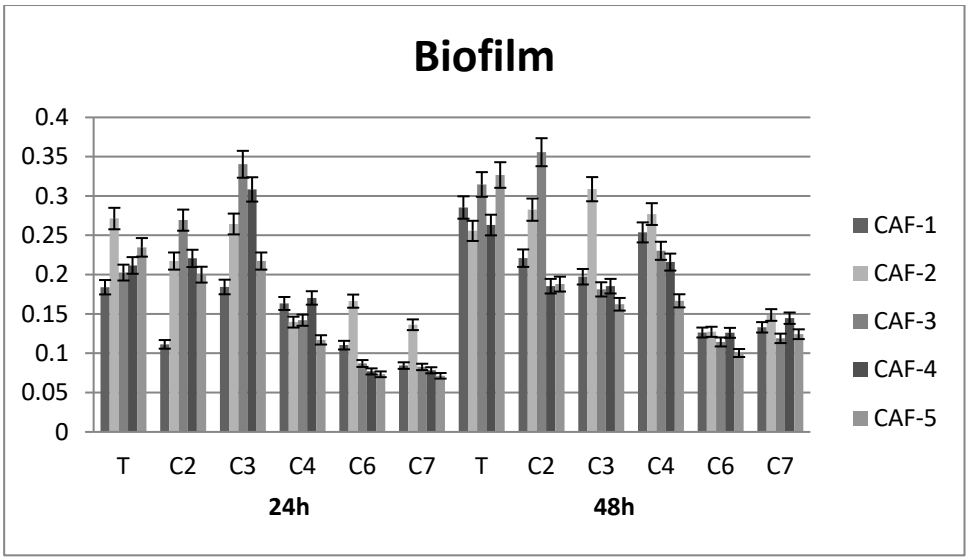

Fig. 4. Impact of different concentrations of glyphosate on biofilm formation after (24-48 hours)

of glyphosate led to a significant increase in biofilm formation compared to the control, up to a concentration of 25 mg/ml (Figure 4). However, beyond this concentration, biofilm formation started to decrease. Furthermore, at 48 hours, biofilm formation was significant in the control group but was inhibited when exposed to 50 mg/ml of glyphosate. Interestingly, under higher doses of glyphosate stress, biofilm development was significantly reduced compared to the control. These results highlight the impact of glyphosate on biofilm formation by the tested strains, with varying effects depending on the concentration and duration of exposure.(Figure. 4)

Glyphosate degradation product

The degradation of glyphosate by microorganisms is typically facilitated through two enzymes pathways: AMPA with the oxidoréductase enzyme and Sarcosine with the C-P lyase enzyme. In our study, HPLC was used to determine the residues of glyphosate degradation by rhizospheric bacterial isolates, after derivatisation with p-toluenesulfonyl, the reaction products, Sarcosine, Glycine, AMPA and glyphosate were ejected at different retention times. The comparison results of the peaks that were detected after approximately 3.64 minutes for CAF-1, 2.85 minutes for CAF-2, 2.59 minutes for CAF-3, 2.72 minutes for CAF-4 and 3.77 minutes for CAF-5 (Figure 5) with the calibration curves allowed us to suggest that the main degradation product of all strains is Sarcosine and Glycine.(Figure.5).

Our study focused on the degradation of glyphosate by bacterial strains isolated from soil, plants, and water, particularly within the Enterobacteriaceae family. Notably, we identified *Enterobacter Coelecae* and *Enterobacter hormaechei* as the strains showing good growth capacity under glyphosate stress, isolated from the rhizospheric soil. To the best of our knowledge, this is the first report of glyphosate degradation by *Enterobacter hormaechei*. Previous studies have highlighted the degradation potential of other *Enterobacter* strains, such as *Enterobacter sp* Bisph1 isolated from Saharan soil in Algeria, which demonstrated efficient degradation at a concentration of 1g/l for 168 hours (Benslama & Boulahrouf, 2013). Kryuchkova et al., (2014) established a correlation between the growth of *Enterobacter cloacae* and the reduction of glyphosate levels in the culture medium, suggesting the utilization of glyphosate as a source of phosphorus (P) and nitrogen (N). Another notable strain, *Bacillus subtilis* Bs-15, as reported by Yu et al., (2015), exhibited glyphosate degradation of 66.97% in sterile soil compared to

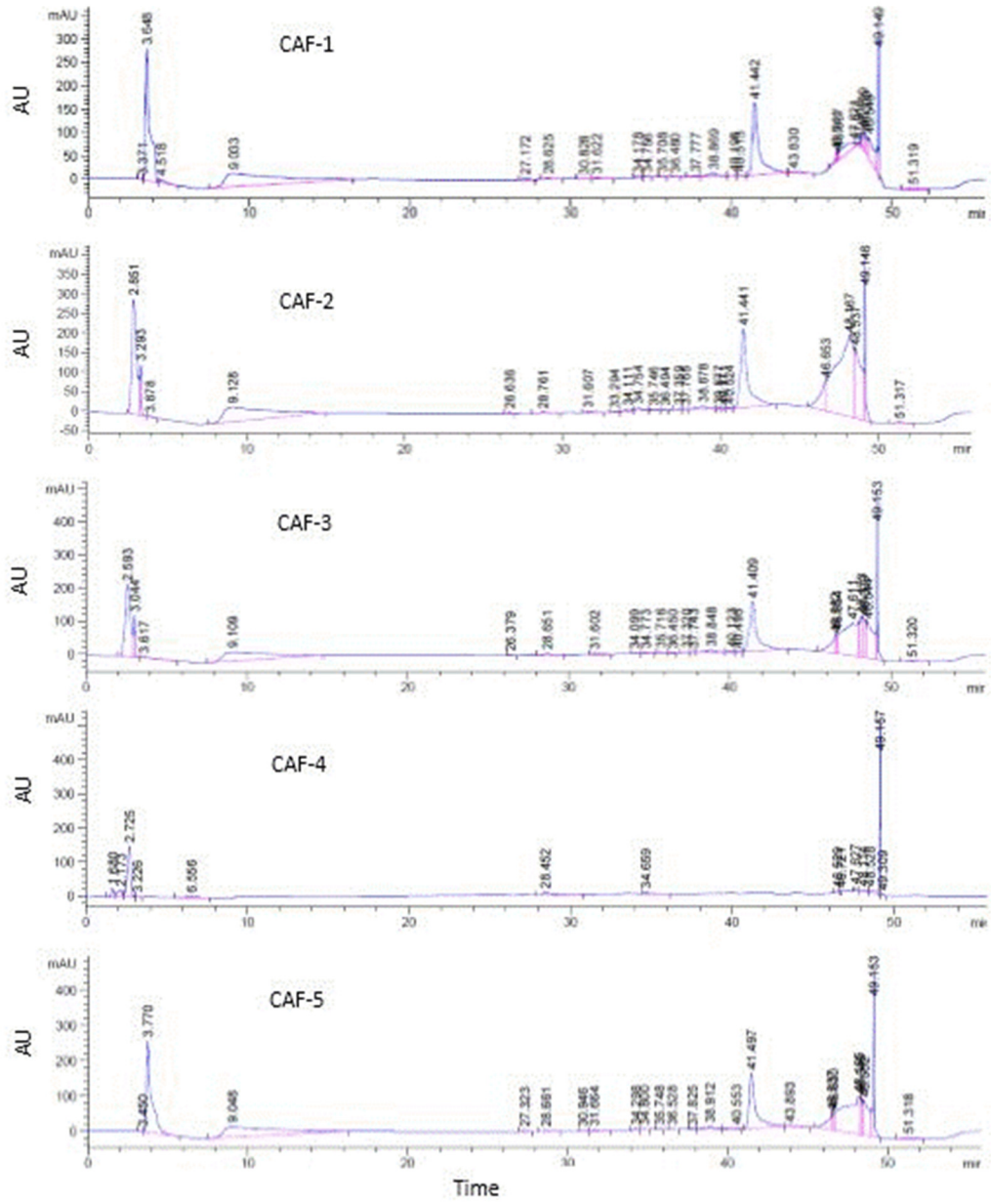

Fig. 5. HPLC chromatograms of biodegradation of glyphosate by bacterial strains and its metabolites GLYP, AMPA, SAR and Glycine after 7 days incubation.

71.57% in contaminated soil. *Acetobacter sp* and *Pseudomonas fluorescens*, studied by Amoros et al., (2007), showed the highest growth in the control group with the lowest glyphosate concentration (7.2 mg/l). In the study by Fan et al., (2012), *Bacillus cereus* CB4 isolated from glyphosate-polluted soil exhibited a remarkable glyphosate degradation capacity of 94.47% within 5 days. Additionally, Firdous et al., (2017) identified *Comamonas odontotermitis* P2, isolated from glyphosate-contaminated soil in Australia, as having the ability to degrade glyphosate (1.5 g/L) within 104 hours. These findings underscore the diverse range of bacterial strains capable of degrading glyphosate and highlight the potential for utilizing specific strains in environmental remediation efforts. In our study, we investigated the degradation capacity of glyphosate by our bacterial strains under different environmental conditions. We found that the growth and degradation of glyphosate were influenced by many factors such as temperature,

pH, and NaCl concentration. The specific optimal conditions may vary depending on the bacterial strain and experimental , the optimal conditions for the growth of *Enterobacter hormaechei* and *Enterobacter cloacae* strains in the presence of glyphosate were determined to be pH 7, 37°C, and specific NaCl concentrations for each strain. Comparing our findings with previous studies, Fan et al., (2012) demonstrated a higher degradation rate of glyphosate (94.3%) by *Bacillus Cereus* (CB4) at 35°C in an acidic environment. On the other hand, Yu et al., (2015) identified *Bacillus subtilis* as a strain that exhibited enhanced degradation at pH 8 and 35°C. Furthermore, Malviya et al., (2015) showed that the optimal NaCl concentration for growth was 1% and pH 7. In contrast, Manogaran et al., (2018) reported that *Burkholderia vietnamiensis* AQ5-12 strain exhibited inhibited growth and degradation of glyphosate at 37°C, 1% NaCl concentration, and slightly acidic pH setup.Rhizosphere soil microbial communities are capable of producing a variety of bioactive compounds, including plant hormones such as auxins, cytokinins, gibberellins and abscisic acids, among others (Shahid et al., 2019). The isolated bacterial strains exhibited PGPR effects, nitrogen fixation, IAA generation, P solubilization, and biofilm formation.The results of our study showed that our strains are capable to form biofilms which are explained by the growth of colonies attached to the surface of the microplate, and the addition of glyphosate inhibits the growth of these bacterial biofilms, this last is composed of extracellular substances containing exopolysaccharides, nucleic acids and proteins and its inhibition may be due to the destruction of the cell membrane caused by environmental stress such as glyphosate. This subsequently modifies permeability and blocks the production of extra-polymeric elements and other virulence factors necessary for the transport of nutrition (Rinaudi et al., 2006; Shahid et al., 2021). A proportional decrease in IAA synthesis and an increase in glyphosate concentration. These results are in accord with those of Andriani et al., (2017), who noted that the plant growth-promoting bacteria *Enterobacter sp*, *Enterobacter cloacae* and *Pseudomonas fluorescens* can degrade the herbicide glyphosate and aid seed germination in glyphosate-containing media. a decrease in phytohormone secretion is linked to a progressive increase in glyphosate concentration (Shahid et al., 2019). Our study showed that the quantity of P-solubilzer in the broth medium decreased with increasing of glyphosate concentrations. These findings align with those of Shahid & Khan, (2018), who studied the impact of glyphosate on the PGPR traits of *Burkholderia cepaciapar*, and Shahid et al., (2021). Soil micro-organisms can metabolise glyphosate in two ways: C-P lyase, which produces phosphate and sarcosine, or glyphosate oxidoreductase, which cleaves the C-N bond to aminomethylphosphonic acid (AMPA). Several studies have been done to determine the degradation and detoxification pathway of the microorganisms. One of these studies was carried out on *Geobacillus caldoxylosilyticus* T20 degrades glyphosate via the AMPA pathway which isolated from central heating system water (Obojska et al., 2002). In another study by Hadi et al., (2013) which isolate *Ochrobactrum sp* to degrade glyphosate to AMPA through the oxidoreductase enzyme. Following *Ochrobactrum intermedium* Sq20 degrades glyphosate to sarcosine and glycine products via C-P lyase (Firdous et al., 2017), Sviridov et al., in 2012 isolated *Ochrobactrum anthropi* GPK3 which degrades glyphosate via both pathways to AMPA, sarcosine glyoxylate and glycine. In 2019, Xu et al., isolated *Providencia rettgeri* GDB 1, which which exhibits the ability to degrade glyphosate to 71.4% through the AMPA pathway. *Chrysaeobacterium sp*. Y16C, degrades glyphosate to AMPA, as reported by Chen et al., (2022).

CONCLUSION

The rhizospheric bacterial strains CAF-1, CAF-2, CAF-3, CAF-4 and CAF-5 isolated from agricultural soil in Algeria were identified as *Enterobacter coelecae* and *Enterobacter hormocheai*, These strains have demonstrated the ability to grow in the presence of glyphosate

and were selected for their potential to degrade glyphosate at optimal conditions. Moreover, they promote plant growth through their PGPR traits such as Nitrogen fixation, Phosphate solubilization, acid indole-3-acetic synthesis and biofilm formation, which promote growth, protect plants and reduce environmental toxicity. Due to these properties, the strains can therefore be involved in the bioremediation of glyphosate-contaminated soils like a Biofertilizers.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript

LIFE SCIENCE REPORTING

No life science threat was practiced in this research

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