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RESEARCH ARTICLE

Validation of bioherbicidal activity of *Kluyvera intermedia* against *Echinochloa crus-galli*

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ABSTRACT

Weeds in rice fields compete with crops for essential resources, causing severe yield losses based on weed infestation levels and control measures. The current study, conducted at Mahatma Gandhi University, Kottayam, Kerala, India, during June 2023 and March 2024, aims to assess the potentiality of *Kluyvera intermedia* as a bacterial biocontrol agent against *Echinochloa crus-galli* (L.) P. Beauv. (barnyard grass), the common weed of rice fields. Of the 127 bacterial isolates, the bacterial strain *Kluyvera intermedia* MA2 efficiently controlled *Echinochloa crus-galli*. The treated plants showed chlorosis after the first 3 days of application of bacteria and complete death of the plant in 7 days. The study's second objective was to identify the active compound responsible for the herbicidal activity of *Kluyvera intermedia*. The characterization of the active compounds via HR LC-MS/MS (Q-TOF) analysis revealed phthalic acid esters, pyrazine derivatives, and quinoline derivatives as bioactive compounds. Molecular docking studies with antioxidant enzymes revealed significant interactions between phthalic acid esters and key amino acid residues: SER173, ARG38, and ALA134 of ascorbate peroxidase; VAL372 and SER374 of glutathione reductase. Propylpyrazine showed strong binding with PRO367 of catalase, ILE93 of glutathione S-transferase, ARG31 of ascorbate peroxidase, and ASP466 of glutathione reductase. Additionally, 6-methylquinoline interacted notably with ALA253 of catalase. Biochemical, enzymatic, and antibiotic profiling identified the bacterium as an IAA-producing, gram-negative and rod-shaped strain. It demonstrated susceptibility to eight antibiotics and the ability to produce several enzymes, including cellulases, catalases, phenylalanine deaminases, and proteases.

Keywords: Biocontrol, *Echinochloa crus-galli*, *Kluyvera intermedia*, Microbial bioherbicide, Rice, Weed management

INTRODUCTION

Bioherbicides include chemical residues derived from natural sources like fungi, bacteria, and plant extracts with herbicidal roles. They could be advantageous over chemical herbicides, as they are a sustainable and environmentally friendly option for weed control (Duke *et al*. 2024). Unlike synthetic herbicides, bioherbicides are biodegradable and less likely to lead to weed resistance. Methyl indole-3-acetate, an auxin analog purified from *Bacillus altitudinis* was successful in suppressing the growth of wild oats (*Avena fatua* L.) (Ma *et al*. 2024). An aromatic polyketide called julichromes isolated from a Streptomyces species showed inhibitory activities against *Amaranthus retroflexus Setaria viridis*, *Portulaca oleracea* and *Chenopodium album* (Ling *et al*. 2023). *Streptomyces gardneri* producing anthraquinone exhibited 100% herbicidal activity against several weeds (Umurzokov *et al*. 2022). *Bacillus weidmannii* obtained from diseased wheat seeds were found to deliver Cry proteins that constrained the growth of ryegrass (Eigharlou *et al*. 2024).

Echinochloa crus-galli (L.) P. Beauv., commonly known as barnyard grass is highly autogamous annual weed in rice paddy fields causing more than 80% loss (Rao 2021). Both *Echinochloa* spp. and rice belong to the family Graminae. Moreover, they share common biological characteristics, nutrient demand, and growth periods resulting in intense competition for resources (Wu *et al*. 2022). The contemporary methods to control this weed include mechanical removal and herbicide use. However, the unjudicious use of herbicides has led to the emergence of herbicide resistance in barnyard grass (Damalas and Koutroubas 2023). The presence of herbicide residues in the environment directly intimidates ecological security and biodiversity.

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Acknowledging these aspects, this study aims to assess the potential of *Kluyvera intermedia* as a bacterial biocontrol agent to control *Echinochloa crus-galli* (L.) P. Beauv.

MATERIALS AND METHODS

Sample collection and isolation of bacteria

The soil samples were collected from different areas in the state of Kerala, India, and outside the State. The collected samples included sandy soils (coastal plains) (Alappuzha, Thiruvananthapuram), alluvium soil (Kottayam), riverine alluvial soils (river banks), laterite soil (Kottayam), black soil (Palakkad), and special group soils (Mangrove soil from Kannur and Desert soil from Rajasthan). Different soil bacteria were obtained by serial dilution and plating on different nutrient media (Nutrient broth, Lura-Bertani broth, King's B broth, and Tryptone Soya Broth; Himedia Laboratories, India). The well-isolated colonies of different bacteria were maintained in the respective culture media slants from which they were obtained and stored at 4^0C for further screening studies.

Screening of isolates for herbicidal activity

Growth of the weeds: Seeds of *Echinochloa crus-galli* were surface sterilized with 70% alcohol for 20 seconds followed by 30-second wash in 3.25% (v/v) NaOCl. The seeds were thoroughly washed with sterile distilled water and given an acid wash with 1N HCl for 30 seconds to break the dormancy and induce germination. The seeds were planted in pots, with one seedling in each pot at a depth of 14cm and filled with solarized soil. The seeds were allowed to germinate and grow into seedlings. Fully grown plants of about 50 cm in height were used for screening purposes.

Assessment of herbicidal activity: Individual colonies of the bacterial isolates were grown in nutrient broth to obtain an optical density of 1 OD at an absorbance of 600 nm. The culture supernatants obtained by centrifugation at 10000 rpm for 10 minutes were used for the treatment. Forty ml of the culture supernatant was treated with the weed plant for 7 days along with control plants treated with sterile distilled water. The plants were observed for morphological changes (chlorosis, necrosis) and finally, complete death as an indication of positive herbicidal activity. A scale by the European Weed Research Council (2010) was used to visually rate the herbicidal effectiveness of different bacteria on the weed plant (Mugehu and Chandiposha 2014) **(Table 1)**. The assessment was done for two seasons, June 2023 and March 2024 at the Mahatma Gandhi University campus in Kottayam, Kerala, India.

Table 1. Ratings as per the European Weed Research Council (2010)

Category number	Percentage of Weed Kill	Herbicidal effectiveness on weeds
1	100	Complete kill
2	97.5-99.9	Excellent
3	95-97.5	Good
4	90-95	Adequate
5	85-90	Just inadequate
6	75-85	Poor
7	65-75	Very poor
8	33-65	Useless
9	0-33	Almost no effect

Herbicide Effectiveness of different bacterial isolates

Name of the isolate	Category number based on the Effectiveness on *Echinochloa cruss-galli*
Pseudomonas sp	5
Streptococcus sp	6
Staphylococcus sp	5
Kluyvera intermedia MA2	2

Identification of potential isolates

Bacterial isolates showing herbicidal activity were identified using biochemical and molecular characterization. The genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The 16S rDNA PCR amplification was carried out in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, U.S.A.) using the primers 16S-RS-F:5′ -CAGGCCTAACACATGCAAGTC-3′ and 16S-RS-R:5′ -GGGCGGWGTGTACAAGGC-3′. The sequencing was carried out in ABI 3500 DNA Analyzer (Applied Biosystems, U.S.A). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1(Kearse *et al.* 2012).

Identification and characterization of active herbicidal component

Growth of weeds: The method for growing and maintaining weed plants is the same as mentione above except that the seeds were allowed to germinate and grow into seedlings in 4-5 leaved stages for experimental use.

Extraction of active compound: Crude metabolites were extracted from the bacterial supernatant by partitioning with a double volume of organic solvents such as hexane, petroleum ether, chloroform, and ethyl acetate in the order of polarity in a separating funnel. The organic phase was collected and dried using a rotary evaporator at 60^0C. The residues collected from each solvent were dissolved in 1% DMSO and used for further analysis for the presence of an active compound.

Testing the efficacy of crude metabolites: The plant seedlings were treated with five replicates of solvent-extracted active components along with suitable controls (distilled water and DMSO) and kept for seven days. The plants were observed for any morphological changes to mark the signs of the herbicide effect. The extract showing promising herbicidal activity was analyzed via HR LC-MS/MS (Q-TOF) to identify the active compound present.

In silico docking studies: The biologically active compounds identified from the crude extract of the isolate were subjected to molecular docking to determine possible interactions with antioxidant enzymes: catalase (PDB ID:5GKN), glutathione-s-transferase (PDB ID:1GNW), ascorbate peroxidase (PDB ID:2XI6), and glutathione reductase (PDB ID:2HQM). The 3D structures of the active compounds were obtained from the PubChem online data server. AutoDock Vina analyzed the molecular interaction between the ligand and the receptor using the PyRx virtual screening tool. The proteins were prepared by AutoDock Tools 1.5.7 (Scripps Research Institute, USA) before docking by removing water molecules and adding polar hydrogens and Kollmann charges. Binding affinities and hydrogen bond interactions were recorded for each enzyme. PyMol (Schrodinger) was used to visualize the crystal structure of complexes.

Characterization of the isolate

Antibiotic profiling: The Kirby- Bauer Disc diffusion test was performed to determine the antibiotic resistance of the test bacteria against 14 antibiotics. Lawn cultured bacterial isolate on Mueller Hinton Agar was treated with different antibiotics, incubated at 30°C for 24 hours, and observed for their zone of inhibition patterns.

Enzyme profiling: The potential isolate was screened for the production of different enzymes including amylase, chitinase, phosphatase, urease, cellulase, catalase, phenylalanine activity, and protease activity by growing them in selective media. Amylase activity was screened on starch medium and after incubation for about 21-24 hrs at 37^0C, the plates were exposed to an iodine solution to check for clear zones around the test bacteria. Chitinase activity was evaluated in the chitin medium and the formation of halo zones was identified as positive for chitinase activity. Phosphatase production was determined in the Sperber medium, continuously checking for clear zones at 48, 72, 120, 144, and 168 hrs respectively due to hydrolysis. A change in the color of the medium from yellow to red was considered positive for urease production. Bacterial inoculated phenylalanine agar slants post incubation are treated with 10% $FeCl_3$ solution and observed for a sudden change in color to green marking a positive reaction. Cellulase activity is screened in a carboxymethylcellulose (CMC-Na) flooded with 0.2% aqueous Congo Red solution and destained by 1M NaCl to see a yellow halo surrounding the bacteria as a positive reaction. The test bacteria were inoculated into a 30% skim milk agar to study protease activity. The formation of clear zones was positive for protease activity.

RESULTS AND DISCUSSION

Biological control is an efficient and environmentally friendly substitute for or in addition to conventional herbicides. Microbial biocontrol agents (BCA) have been created in recent decades for treating bacterial and fungal infections because biological control is an emerging potential alternative. The use of microbial bioherbicides including deleterious rhizospheric bacteria has gained attention for the past many years. Therefore, exploring different bacteria present in different soil types may uncover a novel bioherbicide agent.

Isolation, screening, and identification of potential isolates

Out of the 127 morphologically distinct bacterial isolates obtained, only 4 different bacteria showed positive herbicidal properties against *Echinochloa crus-galli*. They were identified as *Pseudomonas sp*., *Streptococcus* sp., *Kluyvera intermedia* MA2, and *Staphylococcus sp* by molecular characterization. According to the ratings by the European Weed Research Council (2010), *Kluyvera intermedia* strain MA2 showed excellent weed control compared to other bacterial isolates and was selected for further study. The weed plant leaves showed necrosis and chlorosis within 3 days of bioherbicide treatment and the plant was completely damaged after 7 days **(Figure 1)**. The bacteria were identified morphologically, biochemically, and by molecular techniques **(Figure 2. A, B)**. The nucleotide

Figure 1. *Echinocloa crus-galli* plants before and after treatment with *Kluyvera intermedia* MA2.
A. Control: *Echinocloa crus-galli* plants after 7 days of treatment. **B.** Test: *Echinocloa crus-galli* plants before treatment. **C**. Test: *Echinocloa crus-galli* plants after 7 days of treatment with *Kluyvera intermedia* MA2.

sequence of the isolate was deposited in the Genbank with accession number OR399149. The culture has also been deposited in the National Centre for Cell Science (NCCS), Pune, Maharashtra, India (Accession number: MCC5423).

Identification and characterization of active components with herbicidal activity

Microorganisms work in immense ways to alter the soil ecosystem rendering numerous methods for weed control. Many rhizospheric bacteria are found to suppress weed growth by reducing its biomass, and seed production, while some inhibit weeds using their biometabolites. The hexane extracts obtained from *Kluyvera intermedia strain MA2* showed herbicidal activity against *Echinochloa* seedlings. After completing the seven-day experiment, chlorosis and necrosis were consistently seen in the test plants treated with the hexane extract. The absence of any chlorosis and necrosis in the control seedlings ruled out the possibility of the negative action of hexane. The HR LC-MS/MS (Q-TOF) analysis of the *Kluyvera intermedia* strain MA2 extract showed many bioactive compounds. Of these compounds, 6-methylquinoline, propylpyrazine, and phthalic acid mono-2-ethyl hexyl ester are previously reported to have herbicidal properties (Lawrance *et al*. 2019), (Huang *et al*. 2021), (Rybakova *et al*. 2016) **(Figure 3)**. In a previous study quinoline derivatives from *Pseudomonas aeruginosa* H6 showed herbicidal activity against *Pennisetum purpureum*, *Oryza sativa*, *Pisum sativa*, and *Amaranthus spinosum* (Lawrance *et al.* 2019). The herbicidal potential of numerous derivatives of pyrazine, such as analogs of pyrazinamide, and pyrazinoic acid has been established to have 95% control over *Echinochloa crus-galli* (Armel *et al.* 2024). This also supported the action of *Kluyvera intermedia* against *Echinocloa crus-galli* as noted in our study. Physiological studies have revealed that concentration of phthalic acid esters significantly contributed to the increased levels of antioxidant enzyme superoxide dismutase in tobacco plants. Increased antioxidant enzymes suggest oxidative damage in plant systems due to reactive oxygen species. It may indicate that phthalic acid mono-2-ethyl hexyl ester obtained from *Kluyvera intermedia* MA2 might induce oxidative damage in *Echinochloa* plants, rendering their growth inhibition. The utilization of bacterial secondary metabolites for effectively eradicating weeds has proved to be competent in sustainable agriculture. Herbicidal metabolites from *Bacillus velezensis* are efficient in controlling Egyptian broomrape *Orobranche aegyptiaca* (He *et al*. 2022).

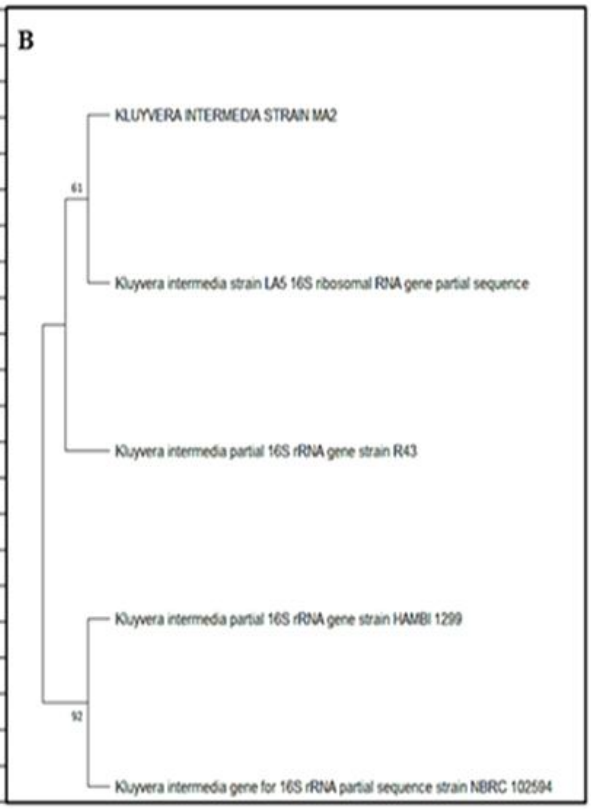
Molecular docking of the bioactive compounds 6-methylquinoline, propylpyrazine, and phthalic acid mono-2-ethyl hexyl ester obtained from *Kluyvera intermedia* strain MA2 revealed the probable interactions of these compounds with the antioxidant enzymes catalase, ascorbate peroxidase, glutathione -s-transferase and glutathione reductase. The ligands phthalic acid mono-2-ethyl hexyl ester and propylpyrazine showed hydrogen bonds with enzymes catalase, glutathione-s-transferase, ascorbate peroxidase, and glutathione reductase. However, 6-methylquinoline showed no interactions with glutathione-s-transferase, ascorbate peroxidase, and glutathione reductase **(Figure 4)**.

The phthalic acid mono-2-ethyl hexyl ester-catalase complex showed H-bond interactions with ASN243, LYS242, and HIS210, while propylpyrazine and 6-methylquinoline complexes with catalase exhibited single H-bond interactions with PRO367 and ALA253 respectively. In the complexes made by both phthalic acid mono-2-ethyl hexyl ester and propylpyrazine with glutathione-s-transferase, one H-bonds were made each by the two ligands with GLN72 and ILE93 of the enzyme. The structural complex of phthalic acid mono-2-ethyl hexyl ester with ascorbate peroxidase showed strong H-bond interactions with the active site residues ARG38 and ALA134 respectively. In molecular docking studies, binding energy is attributed to the strong interaction between a target and a ligand molecule. The more negative the binding affinity is, the stronger the attachment (López-Camacho *et al*. 2016). This study revealed negative binding energy for all the antioxidant enzymes indicating a stronger interaction possibility. The formation of H-bonds also constitutes the global binding energy of the ligand-protein

A

Colony morphology	Size	Medium
	Margin	Irregular
	Opacity	Opaque
	Surface	Wrinkled
	Colour	Creamy (not white)
Cell characters	Gram's reaction	Gram negative
	Shape	Rod
	Motility	Motile
	Endospores	+
Biochemical characters	Indole	-
	Methyl red	+
	Vogues Proskauer	+
	Citrate utilisation	+
	H_2S production	-
	Gelatin Hydrolysis	-
	Oxidase	-
	Catalase	+
	Urease	-
Fermentation	Glucose	+
	Sucrose	+
	Galactose	+
	Sorbitol	+

B



C

Enzyme	Activity
Amylase	-
Chitinase	-
Phosphatase	-
Urease	-
Lipase	-
Cellulase	+
Catalase	+
Phenylalanine deaminase	+
Protease	+

Antibiotics (µg/disc)	Resistant/Sensitive	Zone of Inhibition (mm)
Rifampicin[15]	Resistant	14
Cefipime[30]	Resistant	-
Amikacin[10]	Sensitive	22
Amoxyclav[10]	Resistant	7
Penicillin[10]	Resistant	-
Cefoperazone[75]	Intermediate	18
Tetracyclin[10]	Sensitive	26
Chloramphenicol[10]	Sensitive	21
Gentamycin[10]	Sensitive	26
Erythromycin[15]	Sensitive	28
Streptomycin[10]	Sensitive	23
Ampicillin[10]	Resistant	-
Cefixime[5]	Resistant	-
Imipenem[10]	Sensitive	22

Figure 2. A. Morphological and Biochemical Characteristics of *Kluyvera intermedia* strain MA2. **B**. Phylogenetic tree showing *Kluyvera intermedia* strain MA2 and other similar sequences from the same genus. **C**. Enzyme profile and Antibiotic profile of *Kluyvera intermedia* strain MA2.

Sl No.	Name of the compound	Retention time	Formula	Polarity
1.	6-methylquinoline	5.105	$C_{10}H_9N$	M+ H+
2.	Propylpyrazine	5.596	$C_7H_{10}N_2$	M+H+
3.	Phthalic acid mono-2-ethyl hexyl ester	19.708	$C_{16}H_{22}O_4$	M+H+

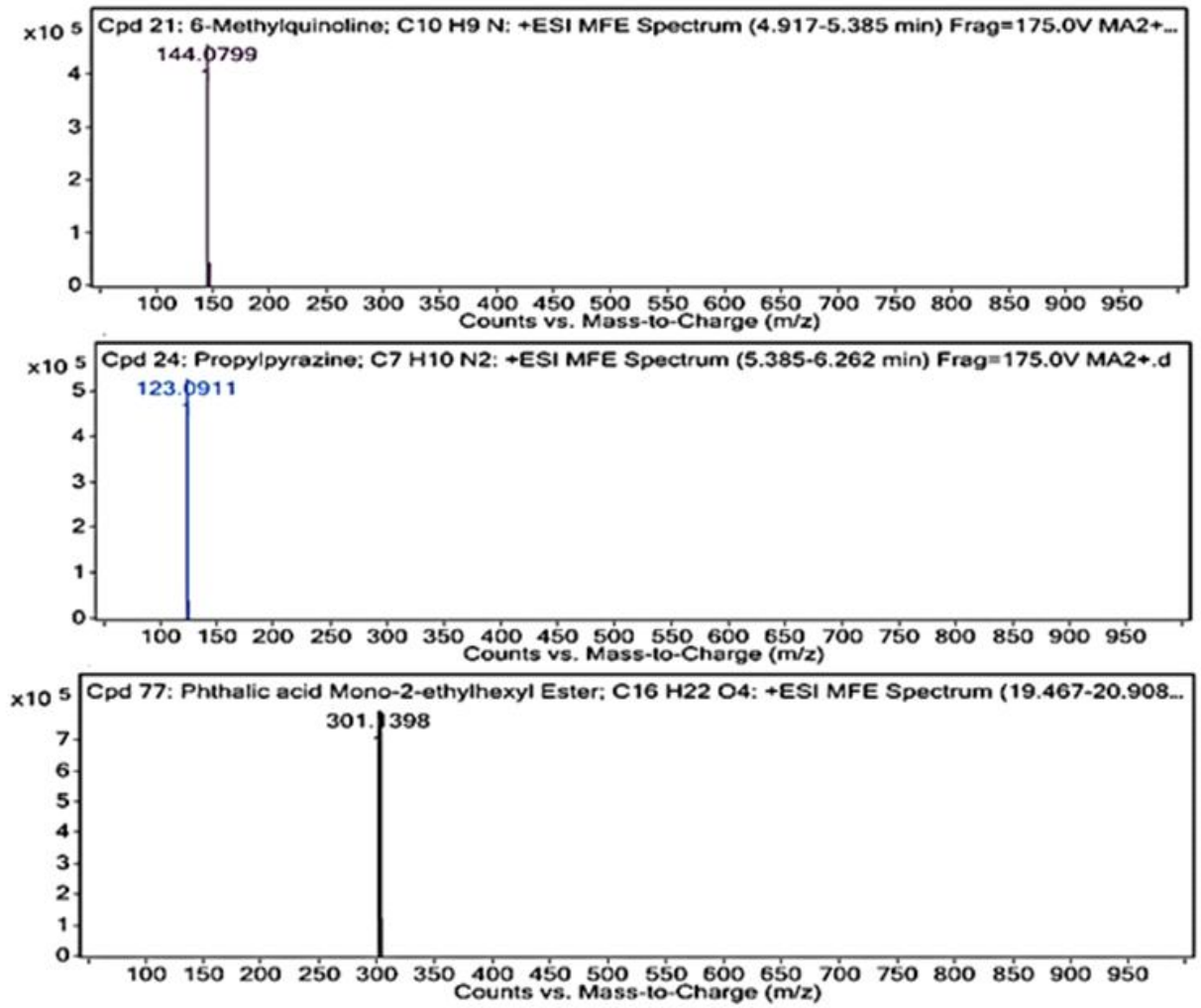


Figure 3. HR LC-MS/MS (Q-TOF) profile of active compounds from *Kluyvera intermedia* MA2 showing bioherbicidal activities

complexes. It was evident from the crystal structures the formation of H-bonds among the various complexes formed between the antioxidant enzymes and the ligands.

Plants constitute a multilevel and intricate system of antioxidant operations to tackle reactive oxygen species in their growth environment (Dumanovic *et al.* 2021). Molecular docking studies can reveal possible conformational changes to the protein and ligand that may disrupt the natural structure of the enzymes by adhering to various amino acid residues. The global binding energy of the antioxidant enzymes with all the bioactive compounds was negative indicating stronger interactions with each other. The highest value was attributed as -8.2 kcal/mol for glutathione-s-transferase-phthalic acid mono-2-ethyl hexyl ester complex. However, the binding energy is low for the same ligand with ascorbate peroxidase but it has stronger H-bond interactions with active site residues ARG38 and

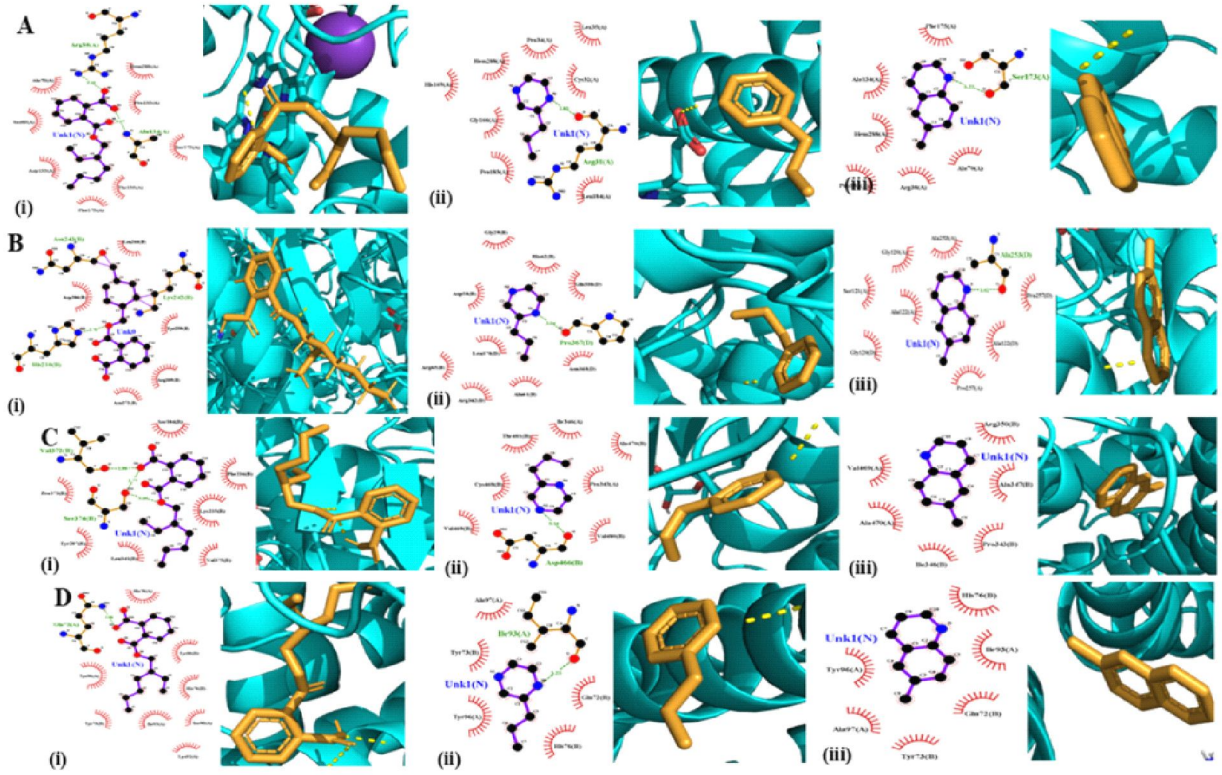


Figure 4. Visualization of bioactive compounds in complex formation with antioxidant enzymes representing 2D and 3D structures. (A) Ascorbate peroxidase, **(B)** Catalase, **(C)** Glutathione reductase, and **(D)** Glutathione-s-transferase [**(i)** phthalic acid mono-2-ethyl hexyl ester, **(ii)** propylpyrazine, and **(iii)** 6-methylquinoline]

ALA134 of the enzyme. 6-methylquinoline also interacts with the active site residues of ascorbate peroxidase at SER173 with a binding energy of -5.5 kcal/mol. Moreover, 6-methyl quinoline does not show any H-bonds with glutathione-s-transferase or glutathione reductase but has a negative binding affinity of -7.2 and -5.9 kcal/mol respectively. Negative binding energy indicates a favorable interaction. However, visualization of the 2D/3D structures reveals no formation of H-bonds with glutathione-s-transferase and glutathione reductase. Even though H-bonds signify a stronger attachment negative binding energy of the ligand with the enzymes suggests that it might be due to weaker hydrophobic interactions, electrostatic interactions, conformational changes of the protein and/or ligand, or Van der Waals forces of attraction (Schiebel et al. 2018). A better understanding of the physiology of antioxidant enzymes can only be gained after monitoring the level of these enzymes in bioherbicide-treated plants for a particular period.

The antibiotic resistance profile of the isolate MA2 revealed that the bacteria were susceptible to seven antibiotics namely Amikacin[10], Tetracyclin[10], Chloramphenicol[10], Gentamycin[10], Erythromycin[15], Streptomycin[10], Imipenem[10], resistant to six of them being Rifampicin[15], Cefipime[30], Amoxyclav[10], Penicillin[10], Ampicillin[10], Cefixime[5], and intermediate with Cefoperazone[75] (all µg/disc). The enzyme profiling of the isolate MA2 detected positive cellulase, catalase, phenylalanine deaminase, and protease activity **(Figure 2.C)**. Antibiotic profiling of any bacteria is relevant as the resistance of organisms to antibiotics can cause severe health hazards and is essential to cognize the susceptibility range of the organism you are handling (Maugeri *et al*. 2019).

The recent advances in biotechnology enable researchers to design or recreate biological enzymes to be utilized in various processes. By understanding the various enzymes a bacteria can produce, new ways for their synthesis might develop (Kieliszek *et al*. 2021). The enzyme profiling of isolate MA2 revealed that it produces cellulases, catalases, phenylalanine deaminases, and proteases. In tandem with the ability of microbes to promote plant growth, the production of metabolites that induce deleterious effects on weed plants has also been reported.

In this era of integrated weed management, biologically based control agents for weeds have gained virtue. These biological products are used directly or in a derived form as bioherbicides. In this

study, aimed at identifying a bacterial biocontrol agent for effective control of *Echinochloa crus-galli*, the *Kluyvera intermedia* strain MA2 was recognized as a bioherbicide against barnyard grass. To our knowledge, this is the first report demonstrating the bioherbicidal property of *Kluyvera intermedia*. Chromatographic studies lead to identifying the active compounds behind the herbicidal action as phthalic acid mono-2-ethyl hexyl ester, propylpyrazine, and 6-methylquinoline. The *in silico* studies demonstrated the active compounds' possible interactions with the antioxidant enzymes catalase, ascorbate peroxidase, glutathione-s-transferase, and glutathione reductase. This suggests the use of the product of *Kluyvera intermedia* as an effective bioherbicide in the future after meticulous studies on their mechanism of action, toxicity on aquatic organisms, soil microflora, cell lines, *etc*.

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