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RESEARCH ARTICLE

Indigenous Enzyme-Producing Microorganisms as Potential Biocontrol Agents Against Weedy Rice Germination

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Abstract

Weedy rice (*Oryza* spp.) remains one of the most challenging constraints in rice production systems due to its genetic similarity to cultivated rice, which limits the effectiveness of conventional chemical control. This study introduces a novel biological strategy based on indigenous enzyme-producing microorganisms capable of selectively suppressing weedy rice during early developmental stages. A total of 66 microbial isolates were obtained from paddy soil and weedy rice soaking water in Ang Thong Province, Thailand. Functional screening identified 42 amylase-producing and 29 cellulase-producing isolates. Selected high-performance strains exhibited substantial extracellular enzyme activities, reaching 30.15 U/mL for amylase (24 h) and 16.79 U/mL for cellulase (36 h). Unlike conventional biocontrol approaches targeting foliar pathogens or post-emergence weeds, this study focuses on enzymatic interference during seed germination and early seedling establishment. Flask-based bioassays demonstrated that microbial inoculation significantly reduced weedy rice germination and seedling vigor, particularly under mixed planting conditions with cultivated rice. Germination of weedy rice declined from 31.67% in non-inoculated controls to 25.00% following microbial treatment. Seedling length decreased from 8.85 to 6.93 cm, and the vigor index was reduced by 38.2%. Importantly, cultivated rice exhibited minimal growth inhibition, indicating a selective suppressive effect. The findings suggest a previously underexplored mechanism of weed suppression mediated by rhizosphere-active extracellular enzymes, potentially disrupting reserve mobilization and early root development in weedy rice. This selective, enzyme-driven inhibition represents a promising alternative to herbicide-dependent systems and provides a mechanistic framework for developing sustainable, microbiome-based weed management strategies. Field validation and metabolomic profiling are warranted to further elucidate the molecular basis of selectivity.

Keywords: Weedy rice, Rhizosphere engineering, Enzyme-mediated inhibition, Amylase, Cellulase, Biological weed control, Seed vigor suppression

Introduction

Weedy rice (*Oryza* spp.) can cause substantial economic losses in rice production. Depending on weed density and environmental conditions, yield reductions of 10% - 60% have commonly been reported, with severe infestations causing losses of more than 80% [1,2]. Because weedy rice is genetically and morphologically similar to cultivated rice, its control using conventional herbicides is difficult and often ineffective [3]. In addition, excessive use of chemical herbicides has raised concerns regarding environmental contamination, herbicide resistance, and potential risks to human health [4]. Therefore, alternative and

sustainable strategies for controlling weedy rice are urgently needed.

Biological control using microorganisms has been increasingly recognized as an environmentally friendly alternative to chemical weed management. Certain microorganisms are capable of suppressing plant growth through the production of bioactive metabolites, enzymes, or other inhibitory compounds that interfere with seed germination, root elongation, or seedling development. Several bacterial and fungal species have been reported to produce phytotoxic metabolites that inhibit weed growth. For example, *Pseudomonas fluorescens* has been reported to produce secondary metabolites such as hydrogen cyanide and phenazine compounds that suppress weed germination and root development [5]. Similarly, *Streptomyces* spp. can produce phytotoxic compounds including herbicidin and bialaphos that inhibit plant growth and have been explored as microbial herbicides [6]. Fungal species such as *Colletotrichum* spp. and *Alternaria* spp. have also been widely investigated as mycoherbicides because they produce host-specific phytotoxins that can effectively suppress target weed species [7]. In addition, certain *Bacillus* species are known to produce a wide range of extracellular enzymes and bioactive metabolites, including lipopeptides (surfactin, iturin, and fengycin) as well as hydrolytic enzymes such as cellulase and amylase, which may contribute to plant tissue degradation or inhibition of seedling growth [8]. These characteristics highlight the potential of soil microorganisms as biological agents for weed suppression and their important role in sustainable agricultural practices. However, the use of *Bacillus* species specifically for controlling weedy rice has been rarely reported. In our report, *Bacillus subtilis* strain OK1101 was shown to produce extracellular enzymes such as amylase and cellulase and exhibited strong inhibitory effects on the germination and seedling vigor of weedy rice, demonstrating its potential as a biological control agent [9].

Microbial enzymes play an important role in plant–microbe interactions and may contribute to the inhibition of seed germination and early seedling development [10]. Enzymes such as amylase and cellulase are involved in the degradation of starch reserves and cellulose components, respectively, which are essential for energy mobilization and structural integrity during seed germination. Several microorganisms are known to produce these extracellular enzymes. For example, *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been widely reported to produce extracellular amylase, which hydrolyzes starch into soluble sugars [11,12]. In addition, cellulase production has been reported in soil bacteria such as *Bacillus subtilis* and *Streptomyces* spp., which are capable of degrading cellulose in plant cell walls [13,14]. The production of these hydrolytic enzymes may interfere with seed germination and seedling development, suggesting their potential application in biological weed control.

Paddy field soils are rich reservoirs of diverse microorganisms adapted to rice-growing environments [15]. Indigenous microorganisms isolated from these soils may possess unique biological activities that are well suited to local agricultural conditions. However, most previous studies have focused on known microbial strains or commercial biocontrol agents, while limited information is available on the isolation and screening of native microorganisms from paddy field soil for their enzyme-producing ability and biocontrol potential against weedy rice. In particular, studies combining enzyme screening with germination inhibition assays under controlled flask conditions remain scarce. Therefore, the objective of this study was to isolate microorganisms from paddy field soil and weedy rice soaking water collected in Ang Thong Province, Thailand, and to screen the isolates for amylase and cellulase producing ability. Selected isolates with high enzyme activity were further evaluated for their growth characteristics, enzyme production under submerged cultivation, and their potential to inhibit weedy rice seed germination under flask conditions. The findings of this study provide fundamental information for the development of microbial-based strategies for sustainable management of weedy rice.

Materials and methods

Sample collection and seed preparation

Soil samples were collected from paddy fields after rice harvesting in Taladmai District, Ang Thong Province, Thailand (GPS: 14.545982, 100.326187). Topsoil samples (0 - 15 cm depth) were randomly collected from a post-harvest paddy field multiple locations within the study area. In addition, soaking

water obtained from rice seeds was collected from the same area. The soaking water was prepared by first surface-sterilizing the rice seeds used in this study with 3% sodium hypochlorite solution. The sterilized seeds were then transferred into 0.85% (w/v) NaCl solution and inoculated with 10% (v/v) bacterial strain (OD.600 nm =1.0), followed by incubation at room temperature for 48 h. A total of 20 soaking water samples were collected from the soaking containers used during the experiment. The water was stagnant (non-flowing) rather than running water. Samples were collected aseptically and used for microbial isolation. After incubation, the liquid obtained from the soaking process was collected and used as the rice seed soaking water. The rice seeds used in this study were collected from paddy fields in Ang Thong Province, Thailand. After collection, the seeds were air-dried and stored in sealed plastic bags to prevent insect contamination until further use. All samples were transported to the laboratory and processed immediately for microbial isolation.

Isolation and identification of microorganisms

Isolation of soil microorganisms from paddy fields

One gram of soil samples were suspended in 9 mL of sterile 0.85% (w/v) NaCl solution and vortexed to obtain a homogeneous suspension. Serial ten-fold dilutions were prepared up to 10^{-6}. Aliquots (0.1 mL) from appropriate dilutions were spread plated onto Nutrient agar (NA) for bacterial isolation and Potato dextrose agar (PDA) for fungal isolation. Plates were incubated at 37 °C for 24 h (for bacteria) and 30 °C for 7 days (for fungi). Colonies with distinct morphology (color, shape, margin, and surface texture) were selected and purified by repeated streak plating. All experiments were performed in triplicate (n = 3).

Microbial isolation from weedy rice soaking water

Weedy rice soaking water (0.5 mL) was mixed with 4.5 mL of sterile 0.85% NaCl solution and serially diluted to an appropriate dilution level. The diluted suspension was spread-plated onto NA (for bacteria) and PDA (for fungi), followed by incubation under the same conditions described above. Morphologically distinct colonies were purified by streak plating for subsequent experiments. All experiments were performed in triplicate (n = 3).

Identification of selected microorganisms

The selected enzyme-producing isolates were identified using both morphological and molecular approaches. Preliminary identification was conducted based on colony morphology and microscopic characteristics. Colony morphology, including color, shape, margin, elevation, and surface texture, was examined after growth on nutrient agar plates. Gram staining was performed to determine the cell type and Gram reaction. Microscopic observation was also conducted to examine cell morphology and the presence of endospores, which are characteristic features of *Bacillus* species.

For molecular identification, genomic DNA was extracted from the selected isolates using a standard bacterial DNA extraction procedure. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal bacterial primers. The PCR products were purified and subjected to DNA sequencing. The obtained sequences were compared with reference sequences available in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) to determine the closest phylogenetic relatives. Isolates showing high sequence similarity with known *Bacillus* species were classified as *Bacillus* spp.

Screening for amylase and cellulase production

Amylase and cellulase production on plate

Purified isolates were cultured on nutrient agar (NA) for bacteria and potato dextrose agar (PDA) for fungi prior to enzyme screening. Enzyme production was evaluated using plate assays on starch agar containing 10 g soluble starch, 2 g peptone, 1 g yeast extract, 1,000 mL distilled water and 15 g agar for amylase activity and carboxymethyl cellulose (CMC) agar containing 4 g carboxymethyl cellulose, 15 g agar, 1,000 mL distilled water for cellulase activity. Each isolate was spot inoculated onto the respective agar medium and incubated at 37 °C for 24 h (bacterial isolates) and 30 °C for 7 days (fungal isolates) until visible colony growth was observed [9]. For amylase activity screening, iodine–potassium iodide (I_2–KI) solution was applied to the surface of starch agar plates

to detect zones of starch hydrolysis. For cellulase activity screening, Congo red solution was applied to CMC agar plates to visualize cellulose degradation. All experiments were performed in triplicate (n = 3). The colony diameter and clear zone diameter were measured. Hydrolysis capacity (HP) was calculated as follows.

$$HP = \frac{clear\ zone\ diameter}{colony\ diameter} \quad (1)$$

Amylase and cellulase production under submerge fermentation

Selected isolates were cultured on nutrient agar (NA) at 37 °C for 24 h. Bacterial colonies were harvested and suspended in sterile 0.85% (w/v) NaCl solution. The suspension was adjusted to an optical density at 600 nm (OD_{600}) of 1.0 (1×10^7 CFU/mL) and used as the inoculum. The inoculum 10% (v/v) was transferred into 90 mL of Production medium (calcium nitrate tetrahydrate ($Ca(NO_3)_2\cdot4H_2O$), 6.6 g; carboxymethyl cellulose (CMC), 5.0 g; yeast extract, 9.5 g; distilled water, 1,000 mL) in a 250-mL Erlenmeyer flask. The cultures were incubated in a shaking incubator at 30 °C and 150 rpm for 36 h. Samples were collected at 6 h intervals to determine cell growth, viable cell counts, and enzyme activities. Cell growth was monitored by measuring OD_{600}. Viable cell counts were determined by serial ten-fold dilution in sterile 0.85% (w/v) NaCl solution, followed by spread plating (0.1 mL) onto NA. The plates were incubated at 37 °C for 24 h, and colony-forming units were expressed as CFU/mL [9]. All experiments were performed in triplicate (n = 3).

Determination of amylase and cellulase activity

Culture medium were centrifuged at 10,000 rpm for 7 min at 4 °C. The supernatant was collected and used as the crude enzyme extract for amylase and cellulase activity assays. Amylase activity was determined using the dinitrosalicylic acid (DNS) method. The substrate consisted of 1% (w/v) soluble starch prepared in 0.1 M sodium phosphate buffer (pH 4.6). Briefly, 0.5 mL of substrate solution was mixed with 0.5 mL of crude enzyme and incubated at 40 °C for 60 min. The reaction was terminated by adding 1.0 mL of DNS reagent. The mixture was then boiled for 5 min, rapidly cooled on ice for 5 min, and the absorbance was measured at 540 nm [9]. Glucose was used to construct the standard calibration curve. Cellulase activity was also determined using the DNS method. The substrate was 1% (w/v) carboxymethyl cellulose (CMC) prepared in 0.1 M sodium phosphate buffer (pH 4.6). The assay procedure, including incubation conditions, DNS reaction, and calculation of reducing sugars, was performed as described for the amylase assay. One unit (U) was defined as the amount of enzyme producing 1 µmol of substrate per min under the assay conditions. All experiments were performed in triplicate (n = 3).

Effect of microbial treatment on weedy rice seed germination in a flask assay

Preparation of microbial suspension

Selected microorganisms (Isolate 5.2) was cultured in nutrient broth (NB) at 37 °C and 150 rpm for 24 h. The cultures were subsequently streaked onto production agar and incubated at 37 °C for 24 h. Bacterial colonies were harvested and suspended in sterile 0.85% (w/v) NaCl solution. The suspension was adjusted to an optical density at 600 nm of 1.0. For each experimental flask, 5 mL of the microbial suspension was mixed with 45 mL of sterile 0.85% (w/v) NaCl solution to obtain a final volume of 50 mL.

Seed surface sterilization

Weedy rice seeds (*Oryza* spp.) used in this study were collected from paddy fields within the study area. Only healthy and uniform seeds, free from visible damage or contamination, were selected to minimize variability in germination during the experiments. Rice and weedy rice seeds were surface-sterilized using a 3% (v/v) sodium hypochlorite solution prepared with sterile distilled water and a small amount of detergent to reduce surface tension. The seeds were treated in a sonication bath for 20 min (2 cycles) to enhance sterilization efficiency. Subsequently, the seeds were rinsed three times with sterile distilled water to remove any residual sterilant.

Experimental design and incubation

The experiment consisted of six treatments, with 20 seeds per flask as follows:

1) rice seeds + sterile water (control);

2) weedy rice seeds + sterile water (control);

3) rice seeds + weedy rice seeds + sterile water (control);

4) rice seeds + microbial suspension;

5) weedy rice seeds + microbial suspension; and

6) rice seeds + weedy rice seeds + microbial suspension.

All treatments were incubated at room temperature under static conditions for 48 h. Each treatment was performed in triplicate (n = 3). Microbial growth was monitored by measuring the optical density at OD_{600} at 0, 24, and 48 h. After 48 h of incubation, the seeds were rinsed with sterile distilled water and transferred onto sterile Petri dishes for germination assessment. Germination was recorded after 7 days. Seedling root and shoot lengths were measured, and the germination percentage was calculated. The seedling vigor index (SVI) was calculated according to Abdul-Baki and Anderson [16] as follows.

$$SVI = \text{Germination percentage} \times [\text{mean root length} + \text{mean shoot length}] \quad (2)$$

Morphological observation of weedy rice seed after degradation

Uniform weedy rice seeds were selected and divided into 2 groups: Untreated seeds (control) and seeds treated with the selected microbial isolate. For the treatment group, seeds were exposed to the microbial suspension under controlled incubation conditions to evaluate the degradative effect of the isolate on seed structures. After incubation, seeds were gently rinsed with sterile distilled water to remove residual microbial cells and debris. The internal microstructure of rice grains was investigated using synchrotron radiation X-ray tomographic microscopy (SR-XTM) at Beamline BL1.2W, Synchrotron Light Research Institute (Public Organization) (SLRI), Nakhon Ratchasima, Thailand which operated at 1.2 GeV and 150 mA. Whole rice grains were mounted vertically on a sample holder and attached onto the rotation stage of BL1.2W's end station. Samples were scanned under monochromatic synchrotron X-ray illumination. During image acquisition, the samples were rotated through 180° with an angular step of 0.3° to obtain a series of projection images for tomographic reconstruction. A 350-µm alumina foil was used as an attenuator to reduce low-energy X-ray and minimize imaging artifacts, resulting in a mean X-ray energy of approximately 11.5 keV. Transmitted X-rays were converted into visible light using a 100-m-thick YAG: Ce scintillator crystal (Crytur, Czech Republic). The visible-light signal were magnified through a lens-coupled X-ray microscope (Optique Peter, France) and recorded using an sCMOS camera (Andor NEO 5.5, Oxford Instruments, UK) with a detector resolution of 2,560×2,160 pixels and 16-bit grayscale depth. The effective pixel size was approximately 3.61 µm. Data preprocessing, background normalization, and tomographic reconstruction were carried out using Octopus Reconstruction software (TESCAN, Gent, Belgium). The reconstructed datasets were subsequently visualized and analyzed using Drishti software packages version 2.6.4 to generate cross-sectional and three-dimensional renderings for evaluation of internal structural characteristics, including fissures, voids, and morphological defects within the endosperm region [17].

Statistical analysis

All experiments were conducted in triplicate. Data are presented as mean ± standard deviation. Statistical differences among treatments were analyzed using one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$.

Results and discussion

Isolation of microorganisms from paddy field soil and weedy rice soaking water

Microorganisms were successfully isolated from paddy field soil and weedy rice soaking water collected in Ang Thong Province, Thailand. Following serial dilution and spread plating, morphologically distinct colonies were observed on nutrient agar (NA) for bacterial isolation and potato dextrose agar (PDA) for fungal isolation. Colonies exhibiting differences in color, shape, margin, and surface texture were selected and purified through repeated streak plating to obtain pure cultures for subsequent analyses. A total of 61 isolates were obtained from paddy field soil and 5 isolates from weedy rice soaking water. Overall, 40 bacterial isolates (NA) and 26 fungal isolates (PDA) were successfully purified (**Table 1**). The isolates were preliminarily classified based on colony morphology, including pigmentation, colony form, and surface

characteristics. Several dominant bacterial isolates were tentatively identified as *Bacillus* spp. based on colony morphology, Gram-positive rod-shaped cells, and endospore formation. All purified isolates were subsequently subjected to enzyme activity screening and biocontrol assays. The higher number of isolates recovered from paddy soil compared with soaking water reflects the greater microbial diversity and biomass typically found in soil environments. Paddy soils are known to harbor diverse microbial communities, particularly members of Firmicutes such as *Bacillus* spp., which are well adapted to fluctuating environmental conditions through endospore formation [18]. Previous studies also reported that microbial community composition differs significantly among soil, water, and rhizosphere compartments in rice ecosystems [19]. Interestingly, weedy rice has been shown to recruit distinct rhizosphere microbial communities with more complex microbial interaction networks than cultivated rice, potentially contributing to enhanced nutrient acquisition and ecological fitness [20,21]. In addition, the functional performance of indigenous microorganisms is strongly influenced by soil physicochemical properties, nutrient dynamics (C-N coupling) and agricultural management practices, which may affect their ecological adaptation and biocontrol potential [7].

Table 1 Number of microbial isolates recovered from paddy field soil and weedy rice soaking water using different culture media.

Sample source	Culture medium	Microorganism type	Number of isolates
Paddy field soil	Nutrient agar	Bacteria	35
Paddy field soil	Potato dextrose agar (PDA)	Fungi	26
Weedy rice soaking water	Nutrient agar	Bacteria	5
		total	66

Screening of amylase- and cellulase-producing microorganisms

All isolates were screened for their ability to produce amylase and cellulase using plate-based assays. Enzyme activity was indicated by the formation of clear zones surrounding the colonies on starch agar following iodine staining and on carboxymethyl cellulose (CMC) agar after Congo red staining. Among the 66 isolates, 41 exhibited positive amylase activity, whereas 29 showed cellulase activity. Enzyme-producing isolates were predominantly bacterial, as reflected by their higher frequency and larger hydrolysis zones on both starch and CMC agar plates (**Figure 1**). Several bacterial isolates demonstrated pronounced extracellular enzyme activity, while filamentous fungi generally exhibited moderate activity. In contrast, the yeast isolates did not show detectable enzyme activity under the tested conditions.

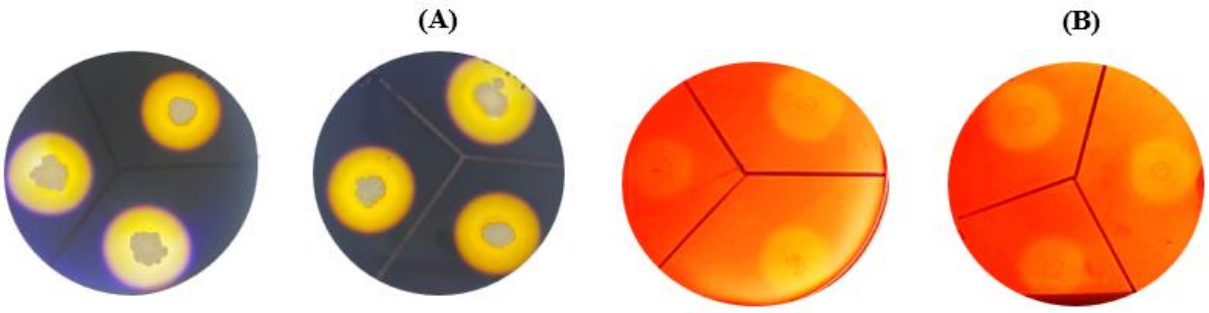


Figure 1 Example of plate assay for extracellular enzyme production by microbial isolates, isolate 5.2: (A) amylase activity on starch agar and (B) cellulase activity on CMC agar after incubation at 37 °C for 24 - 48 h.

Evaluation of hydrolytic activity among enzyme-producing isolates

The hydrolytic capacity (HP), calculated as the ratio of the clear zone diameter to the colony diameter, varied considerably among the enzyme-producing isolates (**Table 2**). For amylase-producing isolates grown on starch agar, HP values ranged from 0 to 3.29, indicating substantial variation in extracellular amylase activity. The reference *Bacillus subtilis* OK1101 exhibited a relatively high amylase activity, with an HP value of 2.85. The highest amylase activity was observed in isolate 5.2 and 9.3 which showed the maximum HP value of 3.29 and 2.89, respectively. For cellulase-producing isolates evaluated on carboxymethyl cellulose (CMC) agar, HP values were generally higher than those observed for amylase-producing isolates. The HP values ranged from 0 to 3.82. Notably, isolates 4.4 and 5.2 exhibited high HP values (>3.5), indicating strong cellulolytic activity and efficient extracellular enzyme secretion. Conversely, isolates with HP values below 3.0 produced small or poorly defined hydrolysis zones, reflecting low cellulase activity. Based on the HP values obtained from both starch and CMC agar assays, isolates demonstrating consistently high hydrolytic capacity were considered promising enzyme producers and selected for subsequent flask-scale cultivation and biocontrol experiments. Isolates exhibiting low or indistinct hydrolysis zones were excluded from further analysis.

Table 2 Hydrolysis capacity (HP) of cellulase and amylase enzyme of bacterial isolates.

Isolate	hydrolytic capacity		Isolate	hydrolytic capacity	
	Cellulase activity	Amylase activity		Cellulase activity	Amylase activity
Bacteria			**Fungi**		
1.1	3.20 ± 0.70^{ab}	1.53 ± 0.10^{d}	**2.2**	0	1.68 ± 0.02^{abc}
1.3	0	1.44 ± 0.15^{d}	**2.3**	3.05 ± 0.83^{ab}	1.40 ± 0.03^{bc}
2.1	3.00 ± 0.38^{ab}	2.65 ± 0.13^{bc}	**2.5**	3.41 ± 0.45^{a}	0
2.2	3.26 ± 0.49^{ab}	0	**4.2**	0	2.48 ± 0.88^{a}
3.4	3.13 ± 0.50^{ab}	2.42 ± 0.33^{bc}	**5.3**	3.04 ± 0.84^{ab}	0
3.6	2.80 ± 0.32^{ab}	2.32 ± 0.35^{bc}	**6.3**	0	1.73 ± 0.14^{abc}
4.1	2.50 ± 0.53^{ab}	2.49 ± 0.12^{bc}	**6.5**	0	1.32 ± 0.05^{bc}
4.2	3.13 ± 0.24^{ab}	1.63 ± 0.40^{bcd}	**7.1**	2.48 ± 0.76^{ab}	1.20 ± 0.04^{c}
4.4	3.66 ± 0.70^{ab}	1.90 ± 0.38^{bcd}	**7.2**	1.91 ± 0.09^{b}	0
4.5	1.9 ± 0.96^{b}	1.82 ± 0.06^{bcd}	**8**	0	2.37 ± 0.81^{ab}
5.2	3.82 ± 0.64^{a}	3.29 ± 0.29^{a}	**9.2**	0	1.91 ± 0.48^{abc}
5.4	1.94 ± 0.85^{b}	2.30 ± 0.86^{bc}	**9.3**	2.79 ± 0.36^{ab}	1.52 ± 0.10^{bc}
7.6	2.43 ± 0.53^{ab}	2.13 ± 0.15^{bc}	**9.4**	0	2.09 ± 0.02^{abc}
7.8	0	1.81 ± 0.10^{bcd}	**10.1**	0	1.28 ± 0.05^{c}
8.2	2.13 ± 0.22^{ab}	2.56 ± 0.20^{bc}	**10.3**	2.71 ± 0.31^{ab}	0
8.4	0	2.09 ± 0.49^{bc}			
9.3	3.12 ± 0.90^{ab}	2.89 ± 0.84^{b}			
9.4	2.53 ± 0.50^{ab}	2.53 ± 0.68^{bc}			

Isolate	hydrolytic capacity		Isolate	hydrolytic capacity	
	Cellulase activity	Amylase activity		Cellulase activity	Amylase activity
Bacteria			**Fungi**		
10.1	3.30 ± 0.75^{ab}	1.84 ± 0.30^{bcd}			
10.4	0	2.19 ± 0.17^{bc}			
OK1101	3.50 ± 0.43^{ab}	2.85 ± 0.53^{b}			

Values are presented as mean ± SD (n = 3). Different letters in the same column indicate significant differences among treatments ($p < 0.05$).

Plate-based screening assays for amylase and cellulase production remain widely used for the preliminary identification of extracellular enzyme-producing microorganisms, as clear hydrolysis zones indicate polysaccharide degradation capability [22,23]. In this study, hydrolytic activity varied considerably among isolates, reflecting differences in genetic and regulatory mechanisms controlling enzyme production [23]. Bacterial isolates generally exhibited stronger enzyme activity than fungi and yeasts, consistent with previous reports highlighting the efficient hydrolytic systems of bacterial taxa such as *Bacillus* spp. [24]. In contrast, no detectable activity was observed in yeast isolates under the tested conditions, likely due to environmental and induction-dependent regulation of enzyme expression [25]. Based on hydrolytic potential (HP) values, three isolates with the highest amylase activity and four isolates with the highest cellulase activity were selected for flask-scale experiments. Among them, isolate 5.2 and 9.3 showed the highest amylase activity on starch agar, while isolates 4.4, 5.2, exhibited strong cellulolytic activity on CMC agar (HP ≥ 3.66), indicating their potential for further submerged cultivation and biocontrol-related applications.

Growth and enzyme production profiles of selected isolates

The growth of selected isolates, including the amylase-producing isolates OK1101, 5.2 and 9.3 as well as the cellulase-producing isolates 4.4, 5.2, and OK1101, was monitored during submerged cultivation in production medium over a 36-h incubation period (**Figure 2**). Cell growth, measured as optical density at 600 nm, increased rapidly during the early incubation phase and reached a maximum at approximately 18 h, followed by a stationary phase with relatively stable OD_{600} values up to 36 h. Viable cell counts exhibited a similar pattern to OD_{600} measurements. The highest colony-forming units (CFU/mL) were recorded between 18 and 24 h of cultivation, depending on the isolate. Notably, isolate 9.3 reached a maximum viable cell density of 10.32 log CFU/mL at 24 h, whereas *B.subtilis* OK1101 attained 9.95 log CFU/mL at 18 h and maintained stable cell numbers thereafter. These results indicate that the selected isolates underwent an active exponential growth phase during the early to mid-incubation period, followed by entry into the stationary phase. The observed growth pattern, characterized by a rapid increase in OD_{600} during the first 18 - 24 h followed by a stationary phase, is consistent with typical bacterial growth kinetics under submerged fermentation conditions. Similar trends have been reported for extracellular enzyme-producing bacteria, particularly species within the genus *Bacillus*, which commonly reach maximum biomass accumulation within approximately 18 - 24 h under optimal culture conditions [26,27]. These studies, highlight that submerged fermentation often promotes rapid biomass development during the logarithmic phase, which is closely associated with primary metabolite production. The strong correlation between OD_{600} values and viable cell counts observed in this study confirms that turbidity measurements reliably reflected microbial proliferation during the exponential phase. Previous reports have demonstrated that OD_{600} measurements are strongly correlated with CFU counts during active growth, although deviations may occur in the stationary phase due to cell aggregation or morphological changes [28]. The peak viable cell densities observed between 18 and 24 h suggest that this time window may represent the

optimal harvesting period for enzyme production. In many extracellular enzyme-producing bacteria, amylase and cellulase synthesis is growth-associated or partially growth-associated, with maximal enzyme secretion occurring during the late exponential or early stationary phase [11,12]. The high cell density achieved by isolate 9.3 (10.32 log CFU/mL) indicates strong adaptation to the production medium and efficient nutrient utilization, which may contribute to enhanced extracellular enzyme secretion. The stabilization of viable counts after 18 - 24 h suggests entry into the stationary phase, possibly due to nutrient depletion or accumulation of metabolic by-products. Stationary phase physiology has been associated with increased secretion of extracellular hydrolases in Gram-positive bacteria as part of stress adaptation and nutrient scavenging mechanisms [29]. Therefore, understanding the timing of growth transitions is essential for optimizing fermentation conditions and maximizing enzyme yield.

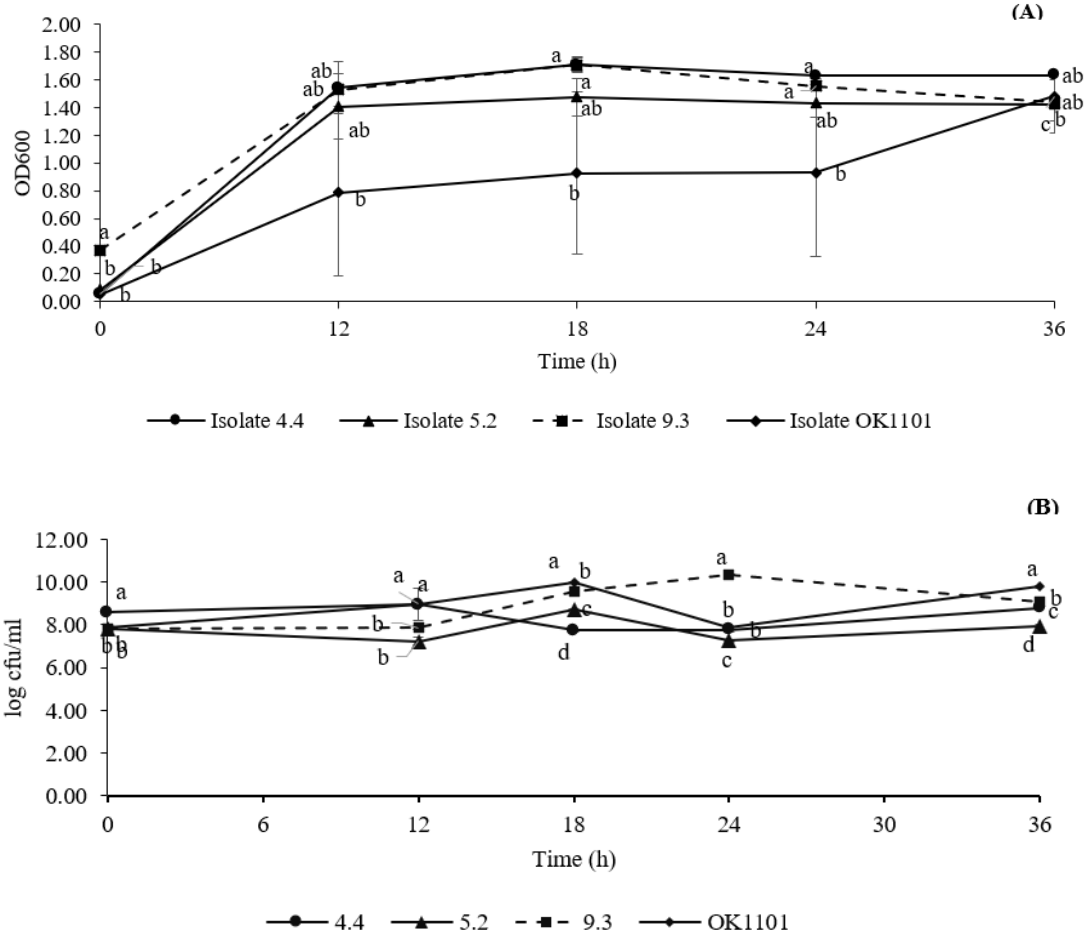


Figure 2 Growth curves of isolates (4.4, 5.2, and 9.3) measured as OD600 (A) and Viable cell counts (log CFU/mL) (B) of selected isolates during submerged cultivation over 36 h. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

Amylase and cellulase activities were determined from crude enzyme extracts collected at 6 h intervals during submerged cultivation (**Figure 3**). Enzyme activity increased with incubation time and reached maximum levels at 24 h for amylase and 36 h for cellulase, depending on the isolate. The highest amylase activity recorded was 30.15 U/mL, observed in isolate 5.2 at 24 h of cultivation. In contrast, the maximum cellulase activity reached 16.79 U/mL, detected in isolate 4.4 at 36 h. After reaching peak activity, a gradual decline or stabilization in enzyme activity was observed in some isolates, particularly during prolonged incubation.

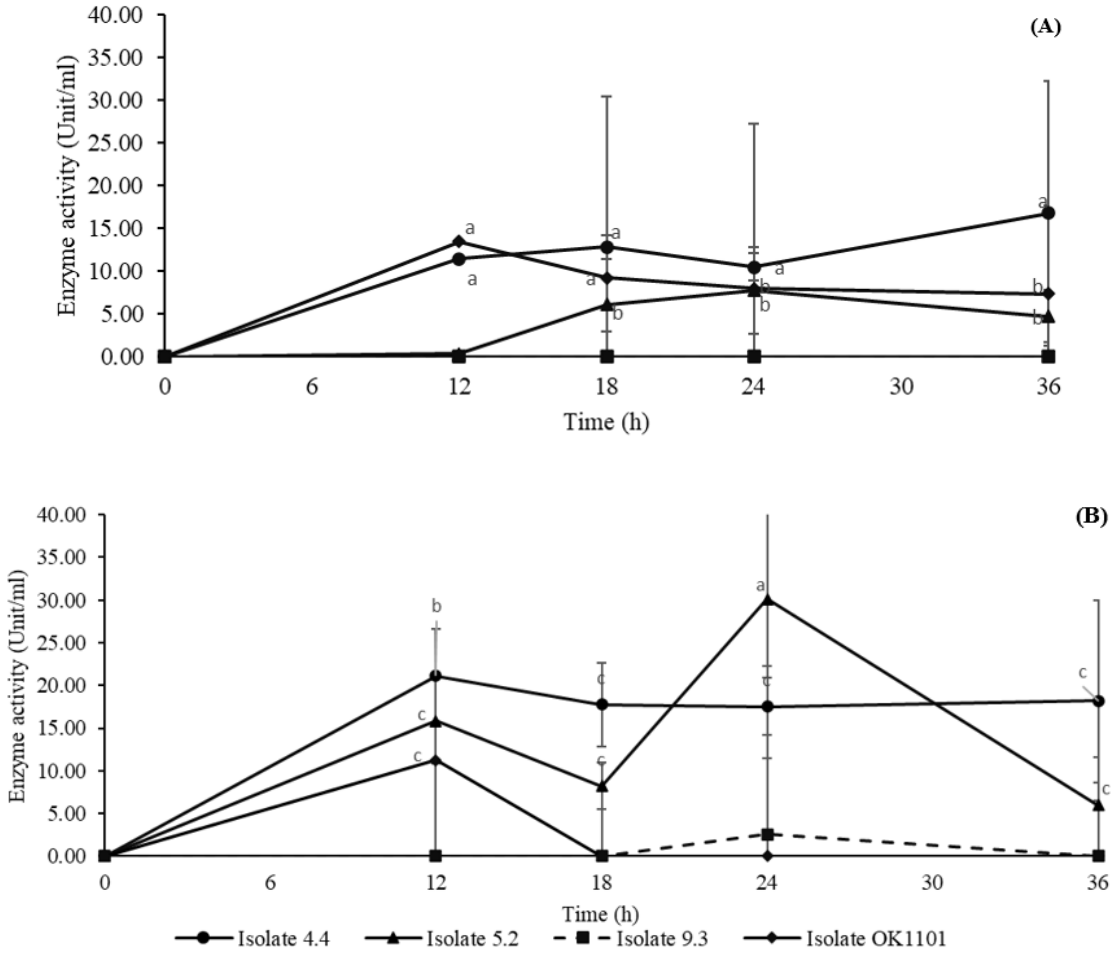


Figure 3 Time-course production of extracellular hydrolytic enzymes by selected microbial isolates during submerged fermentation. (A) Cellulase activity and (B) amylase activity measured at different incubation times (0 - 36 h). Data are presented as mean ± SD. Different letters indicate significant differences among isolates at each time point ($p < 0.05$).

The progressive increase in amylase and cellulase activities during submerged cultivation suggests that enzyme production by the selected isolates was closely associated with active microbial growth. Amylase activity peaked at 24 h, whereas cellulase activity reached its maximum at 36 h, indicating differences in regulatory mechanisms and secretion dynamics between the two enzyme systems. Similar patterns have been reported in bacterial fermentation studies, where amylase is commonly produced as a growth-associated enzyme during the late exponential phase, while cellulase production may extend into the early stationary phase depending on regulatory mechanisms [11,14]. The highest amylase activity (30.15 U/mL) observed in isolate 5.2 at 24 h coincided with maximal biomass accumulation, suggesting that amylase synthesis in this isolate is primarily growth-associated. In many *Bacillus* species, α-amylase production is regulated by carbon source availability and is induced during active starch metabolism [12]. The subsequent stabilization or decline in amylase activity after 24 h may result from nutrient depletion, catabolite repression, or proteolytic degradation of extracellular enzymes during prolonged cultivation. In contrast, cellulase activity peaked at 36 h in isolate 4.4 (16.79 U/mL), suggesting that cellulase synthesis may be partially growth-associated or induced under nutrient-limiting conditions. Cellulase production in microorganisms is often triggered by cellulose or cellulose-derived oligosaccharides and may continue during the transition from exponential to stationary phase [14]. The stabilization or decline of enzyme activity during extended incubation is commonly observed in submerged fermentation systems and may be attributed to reduced metabolic activity,

accumulation of inhibitory metabolites, or enzyme instability in the culture medium [29]. Therefore, identifying optimal harvesting times, 24 h for amylase and 36 h for cellulase in this study is essential for maximizing enzyme yield in subsequent fermentation optimization. Overall, the production profiles observed in the selected isolates demonstrate strong extracellular hydrolytic potential and support their suitability for further biotechnological and biocontrol applications.

Effect of selected microorganisms on germination of weedy rice seeds under flask conditions

After screening for isolates exhibiting the highest enzyme production at the flask level, isolate 5.2 was selected and subsequently used for further experiments on weedy rice inhibition. The growth of the isolate 5.2 during flask incubation was monitored by measuring OD_{600} at 0, 24, and 48 has shown in **Figure 4**. In the non-inoculated control treatments (Paddy, Weedy, and Mix), OD_{600} values remained negligible throughout the incubation period (0.00 - 0.02). In contrast, treatments inoculated with microorganisms (Paddy+, Weedy+, and Mix+) showed markedly higher OD_{600} values, which increased progressively over time. At 24 h, OD_{600} values ranged from 0.16 to 0.18 and further increased to 0.22 - 0.35 at 48 h. These results indicate active microbial proliferation and sustained viability throughout the incubation period.

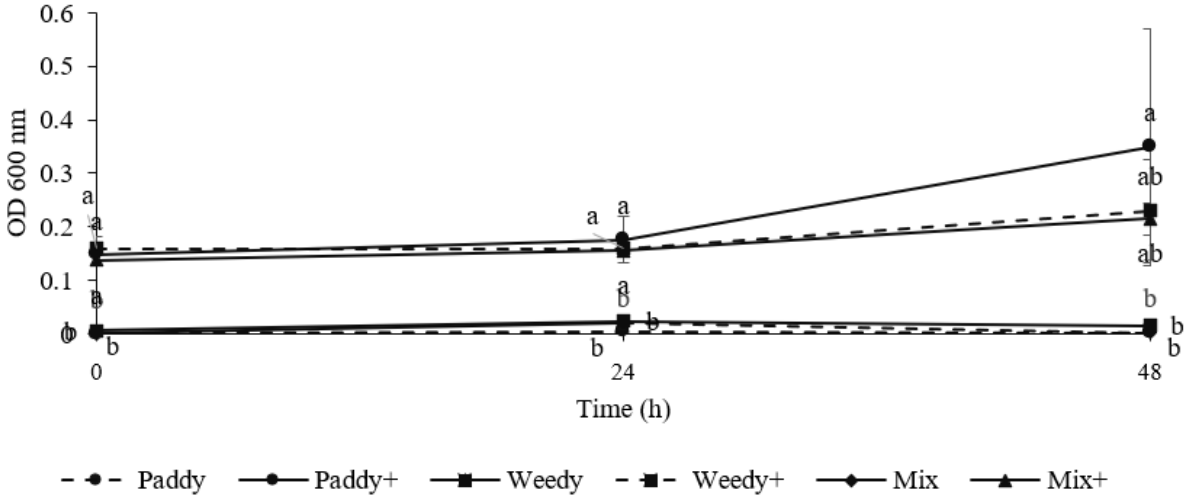


Figure 4 Changes in OD_{600} of microbial suspensions (Isolate 5.2) during flask incubation. Values represent OD_{600} measured from working suspensions prepared by dilution of microbial inoculum (initial OD_{600} = 1.0). Different letters indicate significant differences among treatments ($p < 0.05$).

The germination percentages of rice and weedy rice seeds were evaluated after seven days of incubation. In the control treatments supplied with sterile water, both rice and weedy rice seeds exhibited high germination rates, ranging from 73.33% to 80.00% (**Figure 5**). In contrast, microbial treatments led to a reduction in germination, particularly in weedy rice seeds. The germination percentage of weedy rice treated with microbial suspensions decreased to 76.67%, compared with 80.00% in the control treatment. A more pronounced inhibitory effect was observed under mixed cultivation conditions. When rice and weedy rice seeds were grown together, the germination percentage of weedy rice decreased from 31.67% in the non-inoculated treatment to 25.00% following microbial inoculation. These findings suggest that the selected microorganisms exert a stronger suppressive effect on weedy rice germination in the presence of cultivated rice.

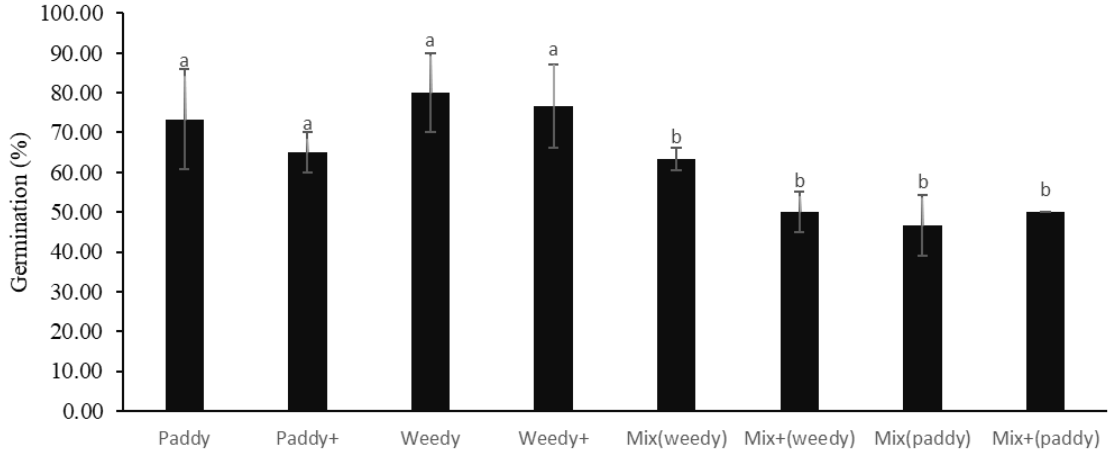


Figure 5 Germination percentage of rice and weedy rice seeds after microbial treatment (48 h). Values are presented as mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

The present study demonstrated that isolate 5.2 significantly suppressed the germination and seedling growth of weedy rice, particularly under mixed cultivation with cultivated rice. The inhibitory effects are likely associated with microbial competition, metabolite production, and interactions with rice-derived allelochemicals that modify the rhizosphere environment [32-34]. Such interactions may enhance the suppression of weedy rice through biochemical and ecological mechanisms, including enzyme production and transformation of allelopathic compounds. Importantly, cultivated rice showed minimal growth inhibition, indicating a selective suppressive effect that supports the potential application of microbial-based weed management strategies. Seedling growth analysis further confirmed that microbial inoculation reduced weedy rice seedling length, especially under mixed planting conditions, while cultivated rice remained largely unaffected. Overall, these findings highlight the potential of microbial inoculation as an environmentally sustainable approach for integrated weedy rice management.

The vigor index was calculated from the data in **Table 3** to evaluate the combined effects of germination percentage and seedling growth. In control treatments supplied with sterile water, weedy rice seedlings exhibited high vigor index values, ranging from 280.46 to 894.26 (**Figure 6**). Microbial treatments caused a substantial reduction in the vigor index of weedy rice seedlings. Under mixed planting conditions, the vigor index of weedy rice decreased to 173.24, representing a reduction of approximately 38.2% compared with the corresponding control (280.46). In contrast, the vigor index of cultivated rice showed no significant reduction or only a slight decrease following microbial treatment, indicating a selective inhibitory effect of the microorganisms on weedy rice seedlings.

Table 3 Effects of microbial treatment on root length, shoot length, and total seedling length after 48 h of incubation.

Treatment	Root (cm)	Shoot (cm)	Total (cm)
Paddy	3.37 ± 0.25	4.02 ± 0.31	7.39 ± 0.56
Paddy + microbes	3.89 ± 0.17	3.99 ± 0.29	7.89 ± 0.37
Weedy	2.88 ± 0.37	7.29 ± 0.34	10.17 ± 0.42
Weedy + microbes	3.81 ± 0.75	6.38 ± 0.50	10.19 ± 0.60
Mix (paddy)	2.81 ± 0.32	3.32 ± 0.30	6.12 ± 0.61
Mix + microbes (paddy)	1.69 ± 0.42	3.06 ± 0.31	4.76 ± 0.73
Mix (weedy)	3.04 ± 0.23	5.81 ± 0.39	8.86 ± 0.59
Mix + microbes (weedy)	1.68 ± 0.52	5.25 ± 2.22	6.93 ± 2.66

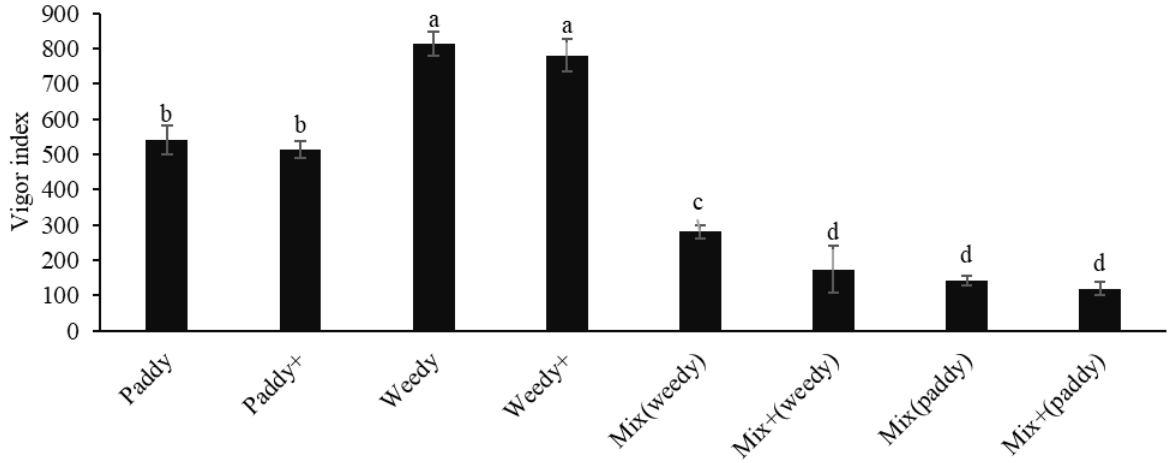


Figure 6 Effects of microbial treatment on the vigor index of rice and weedy rice seedlings (48 h). Values are presented as mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

The present study demonstrated that microbial inoculation significantly reduced germination, seedling growth, and vigor of weedy rice, particularly under mixed planting conditions. The reduction in seedling length and vigor index indicates that microbial suppression extended beyond germination and interfered with early physiological processes such as cell division and elongation. These effects may be associated with microbial metabolites, including organic acids and hydrogen cyanide, which inhibit root development and early weed establishment [31,33]. Stronger suppression under mixed planting further suggests that crop–weed–microbe interactions are highly context-dependent. Rice-derived allelochemicals together with microbial activity may alter rhizosphere conditions in ways that selectively disadvantage weedy rice [30,34]. In contrast, cultivated rice showed only minimal changes in growth and vigor, highlighting the selective nature of the microbial effect and its potential for sustainable biocontrol applications. The suppressive effects are likely mediated through multiple mechanisms, including phytotoxic metabolite production, rhizosphere modification, and interactions with crop-derived allelochemicals. The lower susceptibility of cultivated rice may be related to differences in seed physiology and biochemical defense systems. Previous studies reported that weedy rice exhibits distinct dormancy behavior, rapid germination, and altered reserve mobilization compared with cultivated rice [35], which may increase susceptibility to extracellular hydrolytic enzymes. Cultivated rice may also possess stronger protective barriers, such as seed coat structure, antioxidant systems, and stress-buffering rhizosphere microbial communities, reducing the impact of microbial enzymes and allelochemical stress [36]. Soil minerals and organic acids may further enhance the suppressive activity of *Bacillus* by improving Zn availability, microbial colonization, and nutrient acquisition in the rhizosphere. Organic acids such as malic and citric acids can stimulate zinc-solubilizing bacteria and promote the production of inhibitory metabolites or extracellular enzymes. Therefore, the efficacy of microbial suppression may vary depending on soil physicochemical properties [37]. Under actual paddy field conditions, however, microbial viability and activity may vary with environmental conditions, highlighting the importance of appropriate microbial formulations. Humic acid-amended formulations have been reported to improve the shelf life and survival of plant growth-promoting rhizobacteria under laboratory conditions [38]. Overall, these findings support the potential of microbiome-based approaches as a sustainable strategy for weed management, although further field studies are required to determine microbial persistence, enzyme stability, and suppression efficiency under practical rice cultivation conditions.

Morphological observation of weedy rice seed after degradation

Morphological observations revealed clear structural differences between untreated and microbial-treated weedy rice seeds (**Figure 7**). The untreated seeds (**Figure 7(A)**) retained an intact seed structure, with a continuous and well-preserved seed coat and compact internal tissues. No visible signs of tissue disruption or degradation were observed in the control seeds. In contrast, seeds treated with the microbial isolate exhibited pronounced structural deterioration (**Figure 7(B)**). Visible disruption of the seed coat and internal tissues was observed, including fissures and fragmentation within the endosperm region, as indicated by the arrows. The treated seeds also showed partial collapse and loosening of internal seed structures compared with the untreated control. These structural alterations suggest that the microbial isolate was capable of degrading seed-associated biomolecules and weakening the integrity of the seed tissues. The observed degradative effects may be associated with the production of extracellular hydrolytic enzymes, such as cellulases and amylases, which are known to hydrolyze structural polysaccharides and storage compounds within plant tissues. Degradation of these components could impair seed viability and germination capacity. Overall, the findings demonstrate that the selected microbial isolate possesses significant potential for biological degradation of weedy rice seeds and may represent a promising biocontrol approach for sustainable weed management in rice production systems.



Figure 7 SR-XTM images of rice grains showing external morphology and internal microstructure. (A) Untreated control seed showing intact seed coat and well-preserved internal structures without visible degradation. (B) Seed treated with the selected microbial isolate exhibiting structural deterioration, including disruption of the seed coat and fissures within the internal tissues (arrows), indicating microbial-induced degradation of seed structures. Scale bar = 1,000 μm.

Conclusions

This study successfully isolated indigenous microorganisms from paddy field soil and weedy rice soaking water and demonstrated their ability to produce extracellular amylase and cellulase enzymes. Selected isolates showed strong enzyme-producing potential and exhibited significant inhibitory effects on weedy rice germination and seedling growth under flask-scale conditions. The reduction in germination percentage, seedling length, and vigor index indicates that these microorganisms can interfere with early developmental stages of weedy rice while exerting minimal effects on cultivated rice. The findings highlight the potential application of enzyme-producing microorganisms as environmentally friendly biological control agents for weedy rice management. Although further studies are required to confirm their effectiveness under field conditions, the results of this study provide a scientific basis for the development of microbial-based strategies

aimed at reducing reliance on chemical herbicides and promoting sustainable rice cultivation.

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Declaration of generative AI in scientific writing

The authors acknowledge the use of generative AI tools (ChatGPT) in the preparation of this manuscript, specifically for language editing and grammar correction. No content generation or data interpretation was performed by AI. The authors take full responsibility for the content and conclusions of this work.

CRediT author statement

Chutinun Sengsae: Investigation, Data curation, Formal analysis, Writing original draft, Visualization. **Nantanath Promma:** Investigation, Data curation, Formal analysis. **Ponlkrit Yeesin:** Project administration, Writing – review & editing, **Sukhumaporn Saeng-ngam:** Project administration, Funding acquisition. **Wanlapa Lorliam:** Project administration, Writing – review & editing. **Titiporn Panyachanakul:** Project administration, Writing – review & editing. **Sukhumaporn Krajangsang:** Conceptualization, Methodology, Formal analysis, Writing-review and editing, Supervision, Project administration, Funding acquisition.

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