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Isolation and Characterization of Presumptive *Vibrio* sp. in Milkfish (*Chanos chanos*) and Pond Water for Problem-Based Learning Worksheet Reconstructionof

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Abstract: Microbiology and ecosystem learning in Senior High School (SHS) is often presented abstractly and with limited connections to local environmental issues. This study aimed to isolate and characterize presumptive *Vibrio* sp. from pond water and milkfish (*Chanos chanos*) flesh samples and to analyze their potential for reconstruction as a contextual learning resource in the form of a Biology Student Worksheet (LKPD) for SHS. The research combined qualitative-descriptive laboratory experiments for microbiological testing with descriptive-exploratory analysis of educational potential. Sampling was conducted in a traditional milkfish pond in Pasuruan, East Java, using composite samples from nine water points and nine fish. Isolation was conducted using Alkaline Peptone Salt (APS) enrichment medium and Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar selective medium. Characterization included Gram staining, microscopic observation, and biochemical tests for oxidase, catalase, motility using SIM medium, and TSIA. The results indicated that isolates from pond water and milkfish flesh were presumptive Vibrio sp., characterized by convex yellow/green colonies, Gram-negative curved rod (comma-shaped) cells, motility, and positive oxidase and catalase reactions. TSIA results were A/A for the pond-water isolate and K/A for the milkfish-flesh isolate. These findings are relevant to Phase F SHS Biology Learning Outcomes related to microbiology, pond ecosystems, and food safety. The laboratory findings (micrographs and biochemical profiles) were reconstructed into a Problem-Based Learning (PBL)-based LKPD draft using secondary data, allowing the material to be presented without direct exposure of students to live *Vibrio* cultures (biosafety). This developed LKPD serves as a conceptual framework designed to potentially support scientific literacy and critical-thinking skills. However, as formal expert validation and empirical implementation testing were not conducted, future research is required to evaluate its instructional effectiveness in classroom settings.

Keywords: Biology; Milkfish; Problem-Based Learning; Student Worksheet; *Vibrio* sp.

Introduction

Biology learning at the Senior High School (SHS) level is designed to equip students with an in-depth understanding of scientific concepts and to connect these concepts closely with everyday life (Egara & Mosimege, 2024). However, in practice, microbiology topics, particularly bacterial diversity and the roles of bacteria in ecosystems, are often presented theoretically and abstractly. Learning that focuses primarily on memorizing textual information without relating it to local environmental potential and issues can contribute to low scientific literacy and limited student awareness of their surrounding environment (Purwasih *et al*., 2026). Empirical studies indicate that high school students frequently experience difficulties in visualizing microbial structures and understanding metabolic processes due to a lack of authentic, data-driven instructional media (Qin et al., 2026; Xu & Zhang, 2026). Therefore, innovative learning resources based on local contexts are needed.

Pasuruan Regency is a coastal area with substantial aquaculture potential, particularly milkfish (*Chanos chanos*) farming. Nevertheless, the success of aquaculture activities can be challenged by declining pond-water quality, which may support the

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proliferation of opportunistic pathogenic bacteria such as *Vibrio* sp. (Mary *et al*., 2026; Mutai *et al*., 2026). The presence of *Vibrio* sp. may affect fish health and may also be relevant to food safety for consumers when fish are not properly handled or processed *(Cortés-Sánchez et al*., 2023). The relationship among abiotic conditions (pond-water quality), biotic components (milkfish and *Vibrio* sp.), and potential implications for human health provides a rich case study for integration into Biology learning (Thompson *et al*., 2024). Specifically, raw data from *Vibrio* sp. characterization such as Gram-staining micrographs and TSIA metabolic profiles directly contextualize abstract concepts of bacterial cell morphology, metabolic pathways, and ecosystem interaction dynamics, while highlighting critical biosecurity and food safety issues.

Integrating applied microbiological research data into secondary-school curricula can provide an effective approach for developing authentic Biology learning experiences (Maletici *et al*., 2026). Through exploration of scientific data obtained from the isolation of *Vibrio* sp. from milkfish and pond water, students can learn about microorganism structure and characteristics, interactions among ecosystem components, and environmental pollution issues in a concrete context. This approach is consistent with the emphasis of the Merdeka Curriculum on problem-based learning and the development of students' scientific literacy (Maletici *et al*., 2026). Previous research has explored local environmental issues or literary secondary data for context-based Problem-Based Learning (PBL) worksheets (Utami et al., 2026). However, studies that directly bridge primary microbiology laboratory experiment results into high school PBL worksheets while addressing biosafety considerations remain scarce. This study addresses this gap by transforming primary pathogen characterization data into a biosafety-conscious learning resource.

Although local fisheries research has considerable potential, its use as a Biology learning medium or resource at the SHS level remains limited. Based on this background, this study aimed to: (1) isolate and characterize presumptive *Vibrio* sp. from milkfish (*Chanos chanos*) and pond-water samples based on morphological and biochemical properties; and (2) evaluate and reconstruct these microbiological findings into a contextual PBL-based Student Worksheet (LKPD) for high school Biology topics on microbiology, biodiversity, and ecosystem dynamics.

Method

This study employed a descriptive exploratory laboratory design combined with a pedagogical needs and curriculum mapping analysis for instructional material development. The study was conducted in a traditional milkfish pond located in Blandongan Village, Bugul Kidul District, Pasuruan City, East Java, Indonesia, in December 2025 (Figure 1). Samples were collected from a single pond plot using purposive sampling (Ahmad & Wilkins, 2025). Pond water samples were collected aseptically from nine sampling points at approximately 20 cm below the water surface using sterile bottles. The water samples were then composited aseptically to obtain a composite sample to obtain one composite sample (Tuttle *et al*., 2023). Milkfish (*Chanos chanos*) samples were collected from the same pond plot, with 9 fish collected. The fish flesh was subsequently processed and composited aseptically to obtain one composite flesh sample . All samples were immediately placed in a cool box and maintained at approximately 4 °C using ice packs during transportation to the laboratory (Castro-Morales *et al*., 2024). Microbiological isolation and characterization were conducted at the Fishery Product Safety Laboratory, Faculty of Fisheries and Marine Science, Universitas Brawijaya.

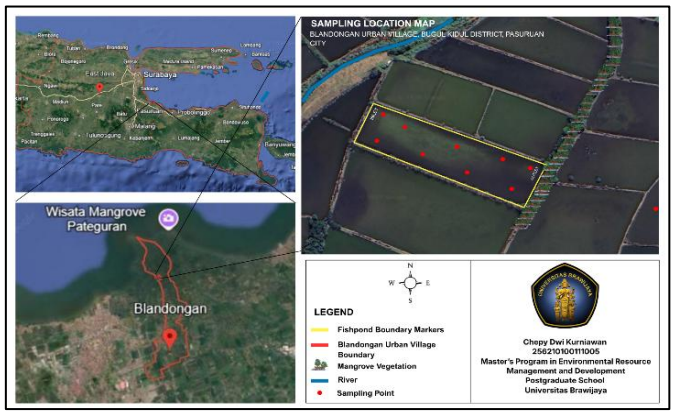

Figure 1. Location in a traditional milkfish pond in Blandongan Village, Bugul Kidul District, Pasuruan City, East Java, Indonesia.

The isolation procedure for *Vibrio* sp. was conducted sequentially using aseptic techniques in analytical duplicates (Haifa-Haryani *et al*., 2022):

1) Aseptic preparation of the work area. The workbench was sterilized by spraying and wiping with 70% alcohol, and the surrounding conditions were maintained aseptically near a Bunsen burner flame.
2) Enrichment and serial dilution. A 25-g portion of milkfish flesh and 25 mL of pond water were aseptically transferred into 225 mL of Alkaline Peptone Salt (APS) solution to obtain a 10^{-1} dilution and homogenized for 2–3 min. Subsequently, 1 mL of the 10^{-1} suspension was aseptically transferred into a test tube containing 9 mL of sterile APS to obtain a 10^{-2} dilution. Both dilution levels were incubated at

36 ± 1 °C for 18–24 h. Changes in turbidity were observed as an indicator of bacterial growth.

3) Selective isolation. Enrichment cultures showing turbidity were collected using a sterile inoculating loop and streaked onto Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar. The Petri dishes were incubated at 36 ± 1 °C for 18–24 h. Presumptive positive colonies were identified based on round yellow colonies associated with sucrose fermentation or green colonies associated with non-sucrose fermentation.

Specific single colonies obtained from TCBS Agar, characterized by yellow or green coloration (Lee *et al*., 2022; Palamae *et al*., 2022), were aseptically transferred using a sterile inoculating loop and purified by streaking on Nutrient Agar (NA) supplemented with 2–3% NaCl (or sterile Nutrient Agar). The plates were incubated at 36 ± 1 °C for 18–24 h until uniform colonies were obtained (Han *et al.,* 2023). Pure colonies on NA were subsequently used for morphological characterization, Gram staining, and biochemical tests and were retained as culture stocks for preparation of secondary data for learning materials. A total of two representative pure isolates (one from pond water and one from milkfish flesh) were selected for subsequent analytical characterization. Given the reliance on phenotypic and biochemical characterization without 16S rRNA molecular sequencing, the isolates are consistently designated as presumptive *Vibrio* sp.

Cell-wall characteristics and microscopic morphology of presumptive *Vibrio* sp. were characterized using Gram staining as follows *(Fazal et al*., 2022; Lainjong *et al.*, 2022):

1) Preparation of bacterial smears. One loopful of a pure colony from NA was aseptically transferred and spread on a glass slide containing sterile distilled water, followed by fixation over a Bunsen burner flame.
2) Staining procedure. The bacterial smear was flooded with crystal violet for 1 min and rinsed with running water. Lugol's iodine solution was then applied for 1 min, followed by decolorization with 96% alcohol for 10–15 s and rinsing. The preparation was subsequently counterstained with safranin for 30 s, rinsed, and air-dried.
3) Microscopic observation. Preparations were observed under a light microscope at 1000× magnification using immersion oil. Presumptive *Vibrio* sp. was indicated by red-stained (Gram-negative), curved rod-shaped (comma-shaped) cells.

Pure presumptive *Vibrio* sp. isolates from NA were subjected to biochemical tests to characterize their physiological properties (Bayhaqi & Insafitri, 2023):

1) Oxidase test. Bacterial colonies were smeared onto filter paper containing oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride). A positive reaction was indicated by a blue-purple color change within 10–30 s.
2) Catalase test. Bacterial colonies were exposed to 3% H_2O_2 on a glass slide. The formation of air bubbles (O2) indicated a positive reaction.
3) Motility and TSIA tests. Isolates were inoculated into SIM (Sulfide Indole Motility) medium to observe bacterial movement and were stabbed and streaked onto TSIA (Triple Sugar Iron Agar) slants to assess sugar fermentation and H2S production.

Laboratory experimental data were analyzed descriptively and qualitatively through the following stages:

1) Curriculum alignment. Microbiological data, including colony morphology, Gram staining, and biochemical characteristics of presumptive *Vibrio* sp., were analyzed in relation to Phase F Biology Learning Outcomes of the Merdeka Curriculum, particularly the elements of Biology Understanding covering Eubacteria/Bacteria, biodiversity, the role of microorganisms in ecosystems, and coastal environments. The suitability of the laboratory data as an instructional resource was systematically evaluated against four criteria: clarity of the study object, alignment with curriculum learning outcomes, clarity of visual/biochemical secondary data, and student learning safety (biosafety).
2) Development of a Student Worksheet (LKPD) draft. Visual data, including photographs of bacterial colonies on selective TCBS medium and microscopic Gram-staining images, together with biochemical-test tables, were synthesized into a contextual learning resource/student worksheet based on the Problem-Based Learning (PBL) syntax. The draft was designed to train SHS students' science-process skills and critical thinking through problems related to pond-water quality as a bioindicator and its implications for coastal environmental health. As a study limitation, the developed LKPD framework represents a conceptual design based on instructional mapping; formal expert validation and empirical classroom testing were not conducted in this study.

Result and Discussion

Isolation using Alkaline Peptone Salt (APS) enrichment medium indicated bacterial growth, as shown by physical changes in the medium, which became turbid after incubation at 36 °C for 24 h (Sanatang et al., 2023). This turbidity indicated the presence of microorganisms capable of utilizing nutrients in the enrichment medium. Inoculation of APS cultures onto selective Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar produced colonies with

characteristics consistent with presumptive *Vibrio* sp. The colony morphology and microbiological characteristics of the purified isolates are presented in Table 1.

Table 1. Colony Morphological Characteristics and Microbiological Properties

Observation/ Test Parameter	Pond-Water Isolate	Milkfish-Flesh Isolate	Biology Concept Indicator
Colony morphology (TCBS)	Round, convex, yellow	Round, convex, green/yellow	Diversity of microbial colony forms
Gram staining	Gram-negative (red)	Gram-negative (red)	Bacterial cell-wall structure
Microscopic cell shape	Curved rod (comma/ *vibrio*)	Curved rod (comma/ *vibrio*)	Basic microorganism morphology
Oxidase test	Positive (+)	Positive (+)	Cell physiology and respiration
Catalase test	Positive (+)	Positive (+)	Cell metabolic enzymes
Motility (SIM)	Positive (+), motile	Positive (+), motile	Locomotion structures (flagella)
TSIA (slant/butt)	A/A (Acid/ Acid)	K/A (Alkaline/ Acid)	Carbohydrate fermentation and metabolism

Gram-staining observations showed that all isolates were Gram-negative bacteria that appeared red and had curved rod-shaped (comma-shaped) cells (Wang *et al.*, 2022; Zermeño-Cervantes *et al.*, 2023). These characteristics are consistent with key morphological characteristics of the family Vibrionaceae (Soto, 2022). Positive oxidase and catalase reactions further supported the characterization of the test isolates as bacteria possessing aerobic/facultatively anaerobic respiratory capabilities and enzymatic activity associated with hydrogen peroxide decomposition (Barua *et al.,* 2023). In addition, positive motility in SIM medium indicated active locomotion, which is associated with flagella (Goh *et al.*, 2026; Vilas Boas *et al.*, 2024).

Testing on Triple Sugar Iron Agar (TSIA) showed differences in carbohydrate fermentation characteristics between the isolates (Uesugi *et al.*, 2024). The pond-water isolate produced an A/A (Acid/Acid) reaction, indicating fermentation of glucose and sucrose and/or lactose in both the slant and butt portions of the medium (Mahmud *et al.*, 2023). In contrast, the milkfish-flesh isolate produced a K/A (Alkaline/Acid) reaction, indicating glucose fermentation in the butt, whereas the slant remained alkaline due to aerobic peptone utilization (Stevens *et al.*, 2024). These differences in biochemical characteristics provide students with a concrete illustration of variation in the physiological properties and metabolic pathways of microorganisms in nature (Brown *et al.*, 2023; Salame *et al.*, 2022).

The identification of presumptive *Vibrio* sp. from both pond water and milkfish flesh reflects the opportunistic nature of these bacteria in marine and aquaculture environments. *Vibrio* species are naturally present in coastal waters, but elevated organic load and fluctuating salinity often trigger their proliferation, posing potential biosecurity risks to aquaculture (Bartkova et al., 2026; Geetha et al., 2026). However, it should be noted that the identification in this study relies on phenotypic and biochemical characterization up to the presumptive genus level. Molecular confirmation (such as 16S rRNA sequencing) would be required for precise species-level identification (Banchi et al., 2022; Liu et al., 2026; Y. Zhang et al., 2026).

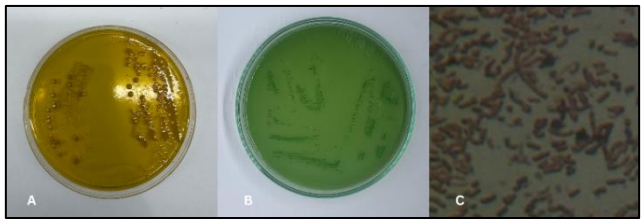

Figure 2. Isolation and microscopic characterization of presumptive *Vibrio* sp.: (A) yellow presumptive *Vibrio* sp. colonies; (B) green presumptive *Vibrio* sp. colonies; (C) microscopic appearance of cells after Gram staining (1000× magnification).

Environmental factors such as high organic matter accumulation from unconsumed feed and fluctuations in water temperature significantly influence *Vibrio* population dynamics in aquaculture ecosystems (Puspitasari et al., 2025; X. Zhang et al., 2024). The presence of these opportunistic bacteria on milkfish flesh highlights a critical food safety concern, as improper handling or undercooking can lead to microbial contamination issues (Taylor et al., 2025). Integrating these ecological and food safety dynamics into the learning material allows students to connect laboratory data with real-world aquaculture problems.

Based on an analysis of the Merdeka Curriculum Biology Learning Outcomes (Phase F), the findings of this laboratory research have potential for reconstruction into a contextual learning resource based on local coastal environmental issue (Pinhassi *et al.*, 2022; Sura *et al.*, 2025). The alignment between the experimental data and SHS Biology curriculum elements is presented in Table 2.

Table 2. Mapping of *Vibrio* sp. Research Findings to Biology Learning Outcomes for Senior High School (Phase F)

Biology Learning Outcome Element	Learning Subtopic/Topic	Integrated Research Data	Application in Learning
1. Biology Understanding : Students understand cell structure and the classification of living organisms.	Microbiology & Classification of Living Organisms	Microscopic Gram-staining images (Gram-negative, *vibrio*-shaped cells) and colony morphology.	Concrete visualization for distinguishing bacterial cell shape and cell-wall characteristics.
2. Biology Understanding: Students identify interactions among ecosystem components.	Ecosystems & Environmental Pollution Issues	Presence of presumptive *Vibrio* sp. in pond water and its occurrence in milkfish.	Case study of the relationship between abiotic factors (pond-water quality) and bacterial population dynamics (biotic component).
3. Scientific Investigation: Students analyze secondary data to draw conclusions.	Food Safety & Applications of Biology	Biochemical characteristics (TSIA, oxidase, catalase) and characteristics of presumptive *Vibrio* sp.	Worksheet activities for analyzing secondary data on microbial contamination hazards and the importance of hygienic food processing.

The suitability of this research as a learning resource was further evaluated using four main criteria for the development of Biology learning media/ materials:

1) Clarity of the learning object. The object under study presumptive *Vibrio* sp. associated with milkfish and pond water is concrete and located within the environment surrounding students in coastal communities, thereby increasing learning relevance through place-based learning.
2) Alignment with curriculum objectives. The laboratory data contain abstract Biology concepts, such as cell metabolism, microbial morphology, and ecosystem interactions, which can become more concrete when presented through real data.
3) Clarity of secondary data. All visual data (colony and microscopic photographs) and qualitative/quantitative biochemical data can be presented clearly in learning materials.
4) Learning safety (biosafety). Because *Vibrio* sp. may include opportunistic pathogenic bacteria, students do not conduct direct practical activities with live cultures. The research is transformed entirely into a learning resource based on secondary-data analysis, making it suitable for classroom use.

From a pedagogical perspective, incorporating authentic laboratory findings into Problem-Based Learning (PBL) bridges abstract biological theory with concrete environmental issues (Rizka et al., 2026). Operationally, each syntax stage in the LKPD targets specific cognitive and science process skills (Sa'adah et al., 2023; Sanudin & Aminatun, 2023). In the Guiding Investigation phase, students analyze raw Gram-staining micrographs and biochemical test profiles (TSIA, catalase, and oxidase), which directly operationalizes science process skills specifically data observation, classification, and interpretation. Furthermore, comparing the physiological characteristics of isolates from different sources (pond water vs. milkfish flesh, such as A/A vs K/A reactions in TSIA) enables students to investigate environmental contamination pathways. During the Developing & Presenting Results stage, evaluating these differential metabolic responses requires students to formulate hypotheses and deduce metabolic pathways. This diagnostic reasoning promotes critical thinking and scientific literacy, as students do not merely memorize bacterial traits but actively evaluate how environmental conditions in aquaculture influence microbial phenotypes and food safety hazards (Khairiyah et al., 2026; Pratiwi et al., 2025).

Based on the curriculum mapping presented in Table 2, the laboratory research data were reconstructed into a contextual Student Worksheet (LKPD) draft based on Problem-Based Learning (PBL). PBL was selected because it can train students' critical-thinking, data-analysis, and problem-solving skills through issues close to their environment (contextual learning) (Loyens *et al*., 2023; Yu & Zin, 2023). The sequence of student activities integrated with PBL syntax is presented in Table 3.

Through this LKPD design, abstract microbiology topics, such as Gram-positive and Gram-negative bacterial cell-wall structure, carbohydrate fermentation in TSIA medium, and cellular respiration and metabolic activity assessed through catalase and oxidase tests, can be presented more concretely to SHS students. Students are not limited to memorizing biochemical terms but are also trained to analyze the ecological functions of bacteria, microbial metabolic pathways, and their implications for ecosystems and the everyday lives of coastal communities. In addition, the use of secondary data derived from laboratory research supports biosafety in school learning. Students can obtain an authentic research-based learning experience without direct exposure to biological hazards associated with live *Vibrio* cultures. Specifically, during the Guiding Investigation phase, analyzing raw Gram-staining micrographs and TSIA test data operationalizes

students' science process skills, particularly data observation and interpretation (Gizaw & Sota, 2023). Subsequently, in the Developing & Presenting Results stage, evaluating differential metabolic pathways fosters critical thinking as students formulate hypotheses regarding aquaculture contamination and food safety.

Table 3. Integration of *Vibrio* sp. Research Data into the Problem-Based Learning (PBL) Instructional Syntax

PBL Syntax	Student Activity	Integrated Research Data	Skill Indicator / Learning Outcome
1. Problem Orientation	Read a narrative article/local case study concerning changes in pond-water quality and their effects on milkfish health in coastal areas.	Issue of presumptive *Vibrio* sp. contamination in pond water and fish tissue.	Problem identification & awareness of food/environmental issues.
2. Organizing Students	Form discussion groups and examine guiding questions about identifying microbial hazards and their potential effects on humans.	Conceptual relationship between environmental quality (abiotic) and bacteria (biotic).	Teamwork & hypothesis formulation.
3. Guiding Investigation	Analyze secondary data from laboratory research presented in tables/figures in the worksheet.	Gram-staining photographs (red, curved rods) and biochemical test data (TSIA, oxidase, catalase).	Scientific data interpretation & data literacy.
4. Developing & Presenting Results	Process biochemical/morphological data to answer worksheet questions and formulate safe post-harvest handling measures for fish.	Physiological characteristics of presumptive *Vibrio* sp. and contamination-prevention methods.	Problem-solving & communication skills.
5. Analysis & Process Evaluation	Present group discussion results to the class and conclude the importance of maintaining pond sanitation and food-processing hygiene.	Synthesis of microbiology, ecosystem, and food-safety concepts.	Critical thinking & reflective thinking.

Compared to previous studies on contextual biology learning that primarily rely on general environmental narratives or secondary data from literature, this research offers a unique contribution by directly integrating primary microbiology laboratory data into high school PBL worksheets (Tobler et al., 2025). By utilizing actual morphological micrographs and biochemical profiles of *Vibrio* sp., the resulting learning media bridges the gap between authentic laboratory research and classroom instruction while strictly maintaining biosafety standards. This provides a concrete model for educators to transform micro-level pathogen research into contextual, inquiry-based learning materials without exposing students to biological hazards.

Despite these contributions, several limitations of this study should be acknowledged. First, the identification of the bacterial isolates was restricted to phenotypic and biochemical characterization at the presumptive genus level, without molecular confirmation such as 16S rRNA sequencing. Second, the developed LKPD framework represents a conceptual design based on instructional mapping; formal expert validation (media and content experts) and empirical implementation testing on high school students were not conducted in this study. Future research should focus on validating the instructional effectiveness of this LKPD in enhancing students' critical thinking and scientific literacy in classroom settings.

Conclusion

Bacterial isolates from pond water and milkfish flesh were presumptively identified as Vibrio sp. based on phenotypic, morphological, and biochemical characterizations. These microbiological data were successfully reconstructed into a contextual biology learning resource aligned with Merdeka Curriculum (Phase F) learning outcomes, focusing on bacterial characteristics, ecosystem roles, and food safety issues. Furthermore, integrating these primary data into a Problem-Based Learning (PBL) worksheet offers a biosafety-conscious conceptual framework designed to support students' scientific literacy and critical-thinking skills. However, as this LKPD framework represents a conceptual design, formal expert validation and empirical implementation testing are required to confirm its instructional effectiveness in classroom settings.

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responsible for the accuracy, interpretation, and integrity of the manuscript.

Author Contributions

Chepy Dwi Kurniawan: Conceptualization, methodology, investigation, formal analysis, data curation, writing – original draft, writing – review and editing, and visualization. The author has read and approved the final version of the manuscript.

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Conflicts of Interest

The author declares that there is no conflict of interest related to the research, authorship, or publication of this article.

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