International Scientific Journal of Engineering and Management (ISJEM) ISSN: 2583-6129
Volume: 04 Issue: 07 | July – 2025 DOI: 10.55041/ISJEM04738
An International Scholarly || Multidisciplinary || Open Access || Indexing in all major Database & Metadata

Isolation, Characterization, and Evaluation of Acid-Tolerant Acetic Acid Bacteria from Rhizosphere Soils for Potential Industrial Vinegar Production

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Abstract
The present study aimed to isolate, characterize, and evaluate acetic acid bacteria (AAB) from diverse rhizosphere soils for their acid production capacity and tolerance, identifying candidates suitable for industrial vinegar fermentation. Ten soil samples were collected from rhizospheres of economically significant crops in Surat, India. Isolates were screened using GYC and Carr agar media, followed by morphological, Gram-staining, and biochemical characterization. Among the ten isolates, three (S1C2, S2C2, and S10) exhibited notable acidogenic activity. Quantitative analysis of acetic acid production in GYE broth revealed that isolate S2C2 produced the highest acetic acid concentration (5.4 g/L) after 72 hours of submerged fermentation. Acid tolerance assays demonstrated that S2C2 maintained appreciable growth at up to 5% v/v acetic acid, outperforming the other isolates. Comparative evaluation with previously reported AAB strains highlighted that the acid productivity and resilience of S2C2 were comparable to or better than established industrial strains. These findings underscore the potential of indigenous rhizosphere soils as reservoirs of robust, high-performance AAB strains, suitable for scalable and sustainable vinegar production processes.

Keywords:
Acetic acid bacteria, Rhizosphere soil, Acid tolerance, Submerged fermentation, Indigenous isolates

1. Introduction

Acetic acid bacteria (AAB) are obligate aerobic, Gram-negative microorganisms widely recognized for their ability to oxidize ethanol to acetic acid, a process central to vinegar production and several other biotechnological applications (Gullo et al., 2009; Zhu et al., 2020). These bacteria predominantly belong to the genera Acetobacter, Komagataeibacter, Gluconobacter, and Gluconacetobacter, and are well known for their role in the fermentation of plant-based and alcoholic substrates (Illeghems et al., 2013; De Roos & De Vuyst, 2018). Beyond vinegar production, AAB contribute to the biosynthesis of gluconic acid, bio-cellulose, and play roles in bioremediation (Raspor & Goranovic, 2008; Zhang et al., 2012; Kanchiswamy et al., 2015).

Traditionally, AAB have been isolated from fermented foods, alcoholic beverages, and fruits; however, recent studies have suggested farm soils and plant rhizospheres as promising untapped niches for novel and metabolically versatile AAB strains (Urbieta et al., 2017; Zhou et al., 2018). Rhizosphere soils are rich in organic matter, plant exudates, and decomposing residues, providing an environment conducive for ethanol-oxidizing and acid-tolerant microorganisms (De Roos & De Vuyst, 2018; Ozturk & Ercisli, 2014). Isolating indigenous strains from these habitats offers potential advantages such as inherent resilience to fluctuating environmental conditions, ethanol concentrations, and elevated acidity levels often encountered in industrial fermentations (Coton et al., 2006; Sengun et al., 2012).

Moreover, identifying new, acid-tolerant, and high-yielding AAB strains holds promise for strengthening local vinegar industries, especially in regions where environmental and economic factors challenge the stability of standard commercial starter cultures (Aydin et al., 2021; Vega et al., 2021). Native strains can offer superior adaptability, enhanced fermentation efficiency, and more sustainable processing, reducing dependency on imported or less resilient strains (Kim et al., 2016).

Given these prospects, the present study was undertaken to isolate, characterize, and evaluate acetic acid bacteria from diverse rhizosphere soil samples collected from agricultural farms in Surat, India. The study focused on assessing acid

production capacity, acid tolerance, morphological and biochemical characteristics of the isolates, with the goal of identifying high-performing strains suitable for industrial vinegar fermentation applications.

2. Material and Methods:

2.1 Soil Sample Collection

Ten soil samples were aseptically collected from the rhizospheric regions of sugarcane, grapes, cotton, aloe vera, neem, sorghum, wheat, castor, fennel, and jamun farms in Surat, India. These samples represented a wide variety of soil types and organic residue contents, potentially supporting diverse microbial populations.

2.2 Media Preparation

The selective isolation of AAB was achieved using two specialized media:

GYC Agar: Composed of Glucose (50 g/L), Yeast Extract (10 g/L), CaCO3 (5 g/L), and Agar (20 g/L). Clear zones around colonies indicated acid production.

Carr Agar: Contained Yeast Extract (30 g/L), Bromocresol Purple (0.02 g/L), Ethanol (2.8 mL/L), and Agar (20 g/L). AAB colonies turned the medium yellow due to acid production.

2.3 Isolation and Screening Acetic acid bacteria

Serial dilutions (10^{-1} to 10^{-6}) of soil suspensions were spread plated on GYC agar and incubated at 30°C for 48 hours. Colonies with clear dissolution zones were indicative of acid production. These colonies were further streaked on Carr agar to confirm acid production via pH indicator colour change. Purified isolates were maintained on GYC slants at 4°C.

2.4 Morphological and Biochemical Characterization

Colony Morphology was observed on nutrient agar after 24-48 hours for size, colour, margin, form, and elevation. The isolates were assessed for their Gram's nature by performing Gram's staining. Further, they were analysed for their standard biochemical characteristics such as catalase production, oxidase production, indole production, methyl red (MR) test, voges–proskauer (VP) test, citrate utilization test, nitrate reduction test, and urease production.

2.5 Acetic Acid Production and Tolerance

2.5.1 Acetic Acid Production

The selected isolates demonstrating strong acidogenic activity during preliminary screening (S1C2, S2C2, S10) were cultivated in Glucose Yeast Extract (GYE) broth to evaluate their acetic acid production capacity. GYE broth was prepared by dissolving Glucose (30 g/L), Yeast Extract (15 g/L), and Ethanol (2.8 mL/L) in distilled water and adjusting the final volume to 1 litre. The medium was sterilized by autoclaving at 121°C for 15 minutes. After cooling, ethanol was aseptically added to the medium.

Each isolate was inoculated into 100 mL of sterile GYE broth contained in 250 mL Erlenmeyer flasks. The flasks were incubated at 30°C on a rotary shaker set at 150 rpm for 72 hours to facilitate aerobic submerged fermentation. At the end of the incubation period, cultures were harvested and centrifuged at 6000 rpm for 10 minutes to separate the bacterial cells.

The concentration of acetic acid produced in the fermentation broth was estimated by titration method. Briefly, 10 mL of culture supernatant was titrated against standard 0.1N NaOH solution using phenolphthalein as a pH indicator. The titration endpoint was marked by the appearance of a persistent pale pink color. The amount of NaOH consumed was recorded, and the acetic acid concentration (g/L) was calculated using the following formula:

Acetic Acid (g/L) = (Volume of NaOH × Normality of NaOH × 60.05 × 1000) / (Sample volume × 1000)

Where 60.05 is the molecular weight of acetic acid.

2.5.2 Acetic Acid Tolerance Assay The tolerance of the selected AAB isolates to different concentrations of acetic acid was assessed by culturing them in GYE broth supplemented with incremental concentrations of acetic acid (2%, 4%, 6%, and 8% v/v). GYE broth was prepared as previously described, with sterile acetic acid added aseptically to achieve the desired concentrations.

Each 100 mL volume of GYE broth containing respective acetic acid concentrations was inoculated with 1 mL of a 24-hour-old culture of each isolate, adjusted to an optical density (OD600) of approximately 0.5 to standardize the inoculum size. The inoculated flasks were incubated at 30°C with shaking at 150 rpm for 72 hours.

Growth of the isolates at each acetic acid concentration was monitored visually by noting turbidity in the broth and confirmed by measuring optical density at 600 nm using a UV-Visible spectrophotometer. The highest concentration at which visible growth and measurable turbidity were observed after 72 hours was recorded as the acetic acid tolerance limit for each isolate. This evaluation determined the robustness of the isolates for potential use in industrial high-acidity fermentation processes.

3. Results

3.1 Isolation and Preliminary Screening

A total of ten rhizosphere soil samples collected from various agricultural crops yielded ten distinct bacterial isolates when cultured on GYC agar medium. The presence of clear halo zones around colonies, formed due to the dissolution of $CaCO_3$, served as a primary indicator of acid production. Among these, eight isolates exhibited distinct, measurable clear zones, suggesting their potential as acidogenic bacteria (Figure-1). These colonies were subsequently sub-cultured on Carr agar medium, which contains bromocresol purple as a pH indicator. Acid production was visually confirmed by a colour change from purple to yellow around the colonies, indicating acidification of the medium. This phenotypic characteristic qualified them as presumptive acetic acid bacteria (AAB) (Figure-2).

ISSN: 2583-6129
DOI: 10.55041/ISJEM04738

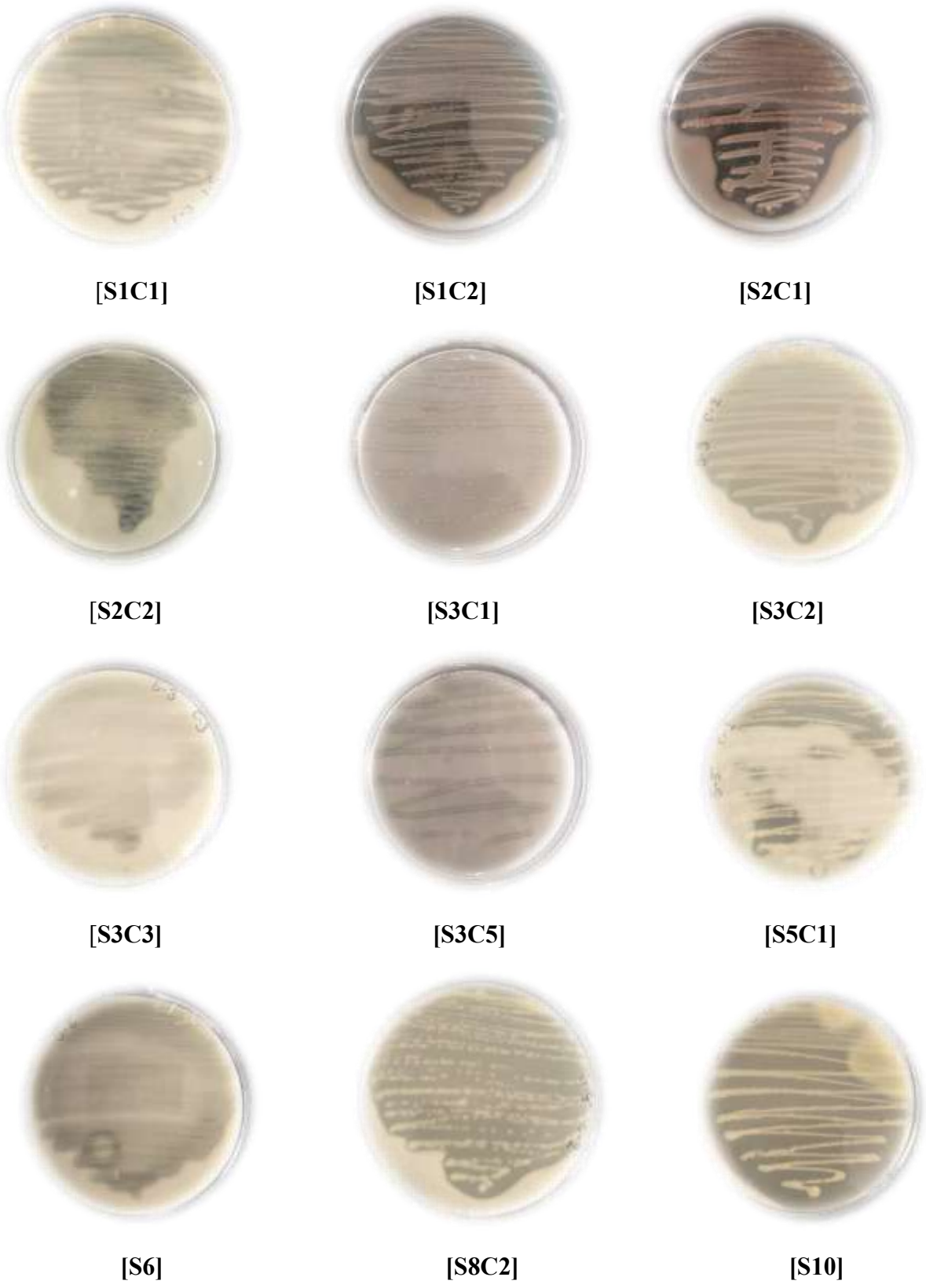

Figure 1 Growth of bacteria on GYC agar media and presence of clear zones around the colonies.

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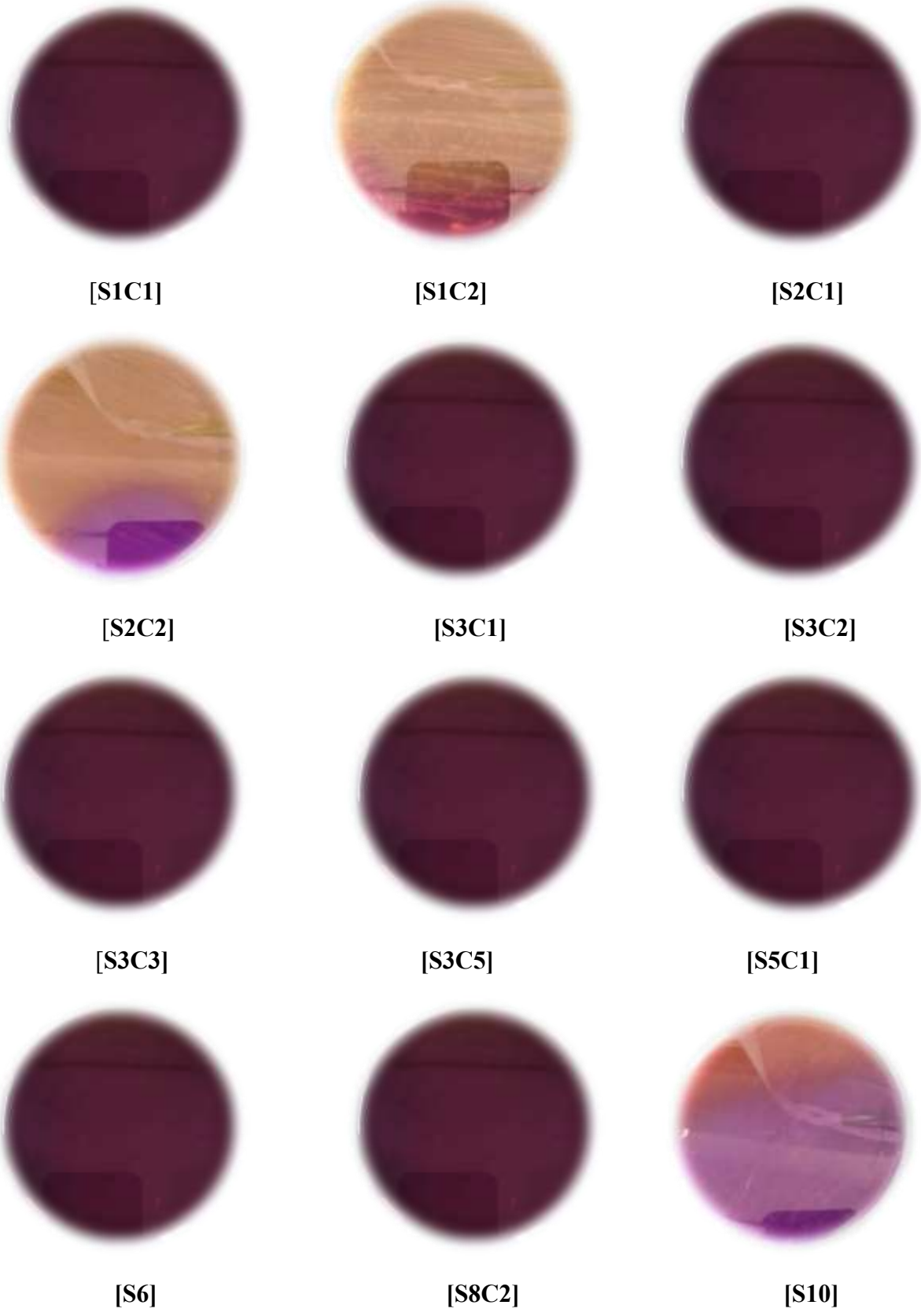

Figure 2 Growth of isolates on Carr agar media.

3.2 Morphological Characterization

Detailed morphological characterization of the isolates was conducted on nutrient agar. The colonies exhibited varied pigmentation, though most were off-white to pale cream, with circular, convex appearances and entire, smooth margins (Table-1). Microscopic examination (Figure-3) after Gram's staining revealed that all eight acid-producing isolates were Gram-negative, short to medium-sized rods (Table-2). This morphological profile corresponded with classical descriptions of AAB belonging to genera such as *Acetobacter, Gluconobacter, and Komagataeibacter.*

Table 1 Colony characteristics of the isolates on Nutrient Agar plate

Isolates	Size	shape	edge	elevation	texture	Consistency	pigment	opacity
S1C1	Small	Round	Entire	Convex	Smooth	Moist	White	Opaque
S1C2	Small	Round	Entire	Convex	Smooth	Moist	White	Opaque
S2C1	Small	Round	Entire	Convex	Smooth	Moist	White	Opaque
S2C2	Small	Round	Entire	Convex	Smooth	Moist	Creamy	Opaque
S3C1	Small	Round	Entire	Convex	Smooth	Moist	White	Opaque
S3C2	Small	Round	Entire	Convex	Smooth	Moist	Creamy	Opaque
S3C3	Small	Round	Entire	Convex	Smooth	Moist	White	Opaque
S3C5	Small	Round	Entire	Convex	Smooth	Moist	White	Opaque
S5C1	Small	Round	Entire	Convex	Smooth	Moist	No Pigment	Opaque
S6	Small	Round	Entire	Convex	Smooth	Moist	Creamy	Translucent
S8C2	Large	Round	Entire	Umbonate	Rough	Dry	White	Opaque
S10	Small	Irregular	Wavy	Flat	Smooth	Moist	White	Opaque

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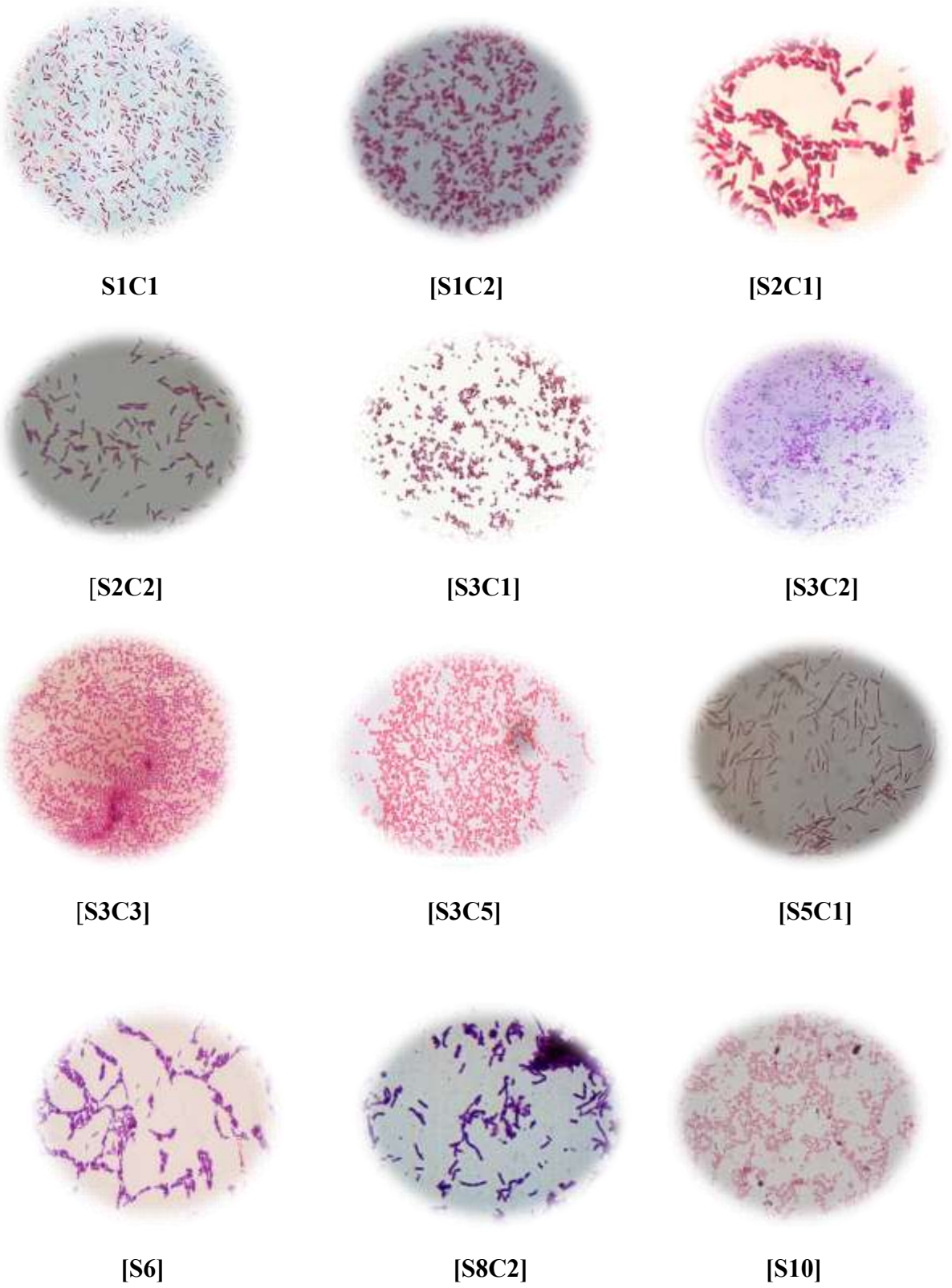

Figure 3 Gram's staining of the isolates

Table 2 Morphological characterisation based on Gram's nature.

Sample	Size	Shape	Arrangement	Cell color	Background color	Gram staining
S1C1	Small	Rod	Single	Pink	White	Negative
S1C2	Small	Rod	Cluster	Pink	White	Negative

S2C1	Long	Rod	Chain	Pink	White	Negative
S2C2	Small	Rod	Single/chain	Pink	White	Negative
S3C1	Small	cocci	Cluster	Pink	White	Negative
S3C2	Small	Rod	Single	Purple	White	Positive
S3C3	Small	cocci	Cluster	Pink	White	Negative
S3C5	Small	Rod	Single/cluster	Pink	White	Negative
S5C1	Long	Rod	Single/chain	Purple	White	positive
S6	Small	Rod	Chain	Purple	White	Positive
S8C2	Small	Rod	Chain	Purple	White	Positive
S10	Small	Rod	Single	Pink	White	Negative

3.3 Biochemical Characterization

All the isolates were subjected to a series of biochemical tests and results are mentioned in Table-3. Amongst them, three isolates (S1C2, S2C2, S10) tested positive for catalase and oxidase, indicating the presence of aerobic metabolism pathways and reactive oxygen species detoxification mechanisms. Positive methyl red (MR) tests confirmed their capability for mixed acid fermentation, while positive citrate utilization demonstrated their ability to use citrate as a sole carbon source. None of the isolates produced indole or urease, nor did they give a positive Voges-Proskauer (VP) reaction. All three strains were positive for nitrate reduction, consistent with known AAB metabolic profiles (Patel & Patel, 2016; Illeghems et al., 2013).

Table 3 Biochemical Characteristics of isolates

SAMPLE	Lactose	Sucrose	Maltose	Glucose	Mannitol	Xylose	Indole	Citrate	TSI	Catalase	Oxidase
S1C1	+	+	-	+	+	+	+	+	Slant- red Butt- red Gas- + H2s- -	-	+
S1C2	+	-	+	+	+	+	-	+	Slant- red Butt- red Gas- + H2s- -	+	-
S2C1	+	+	+	-	+	+	+	+	Slant- red Butt-yellowGas- + H2s- -	-	-
S2C2	+	-	+	+	+	+	-	+	Slant- red Butt- red Gas- + H2s- -	+	-
S3C1	-	+	+	+	+	+	-	+	Slant- red Butt- red Gas-- H2s- -	-	-
S3C2	+	-	+	+	+	+	+	+	Slant- red Butt- red Gas- - H2s- -	-	-
S3C3	+	+	+	-	+	+	+	+	Slant- red Butt- red Gas- - H2s- -	+	-
S3C5	+	+	+	-	+	+	+	+	Slant- red Butt- red Gas- + H2s- -	-	-
S5C1	+	+	-	+	+	+	+	+	Slant- red Butt- red Gas- + H2s- -	+	+
S6	+	+	+	+	-	+	-	+	Slant- red Butt- red Gas- + H2s- -	+	+

S8C2	-	+	+	+	+	+	-	+	Slant- red Butt- red Gas- + H2s- -	+	+
S10	+	-	+	+	+	+	-	+	Slant- red Butt- red Gas- + H2s- -	+	-

3.4 Acetic Acid Production in GYC agar and GYE Broth

Quantitative evaluation of acetic acid production by the three selected isolates was conducted by culturing them in GYE broth under submerged fermentation conditions for 72 hours at 30°C with shaking at 150 rpm. The concentration of acetic acid in the culture supernatant was estimated through titration using 0.1N NaOH.

The results revealed considerable variation in acid production among the isolates. Isolate S2C2 demonstrated the highest acetic acid yield, producing 5.4 g/L of acetic acid. Isolate S1C2 followed closely with 5.0 g/L, while isolate S10 produced 4.2 g/L. The high productivity observed in isolate S2C2 is particularly significant as it matches or surpasses the typical acetic acid yields achieved by many industrially used strains under similar fermentation conditions (Gullo et al., 2009). This confirmed the potential of these rhizosphere-derived isolates as efficient acid producers suitable for industrial vinegar fermentation processes.

ISSN: 2583-6129
DOI: 10.55041/ISJEM04738

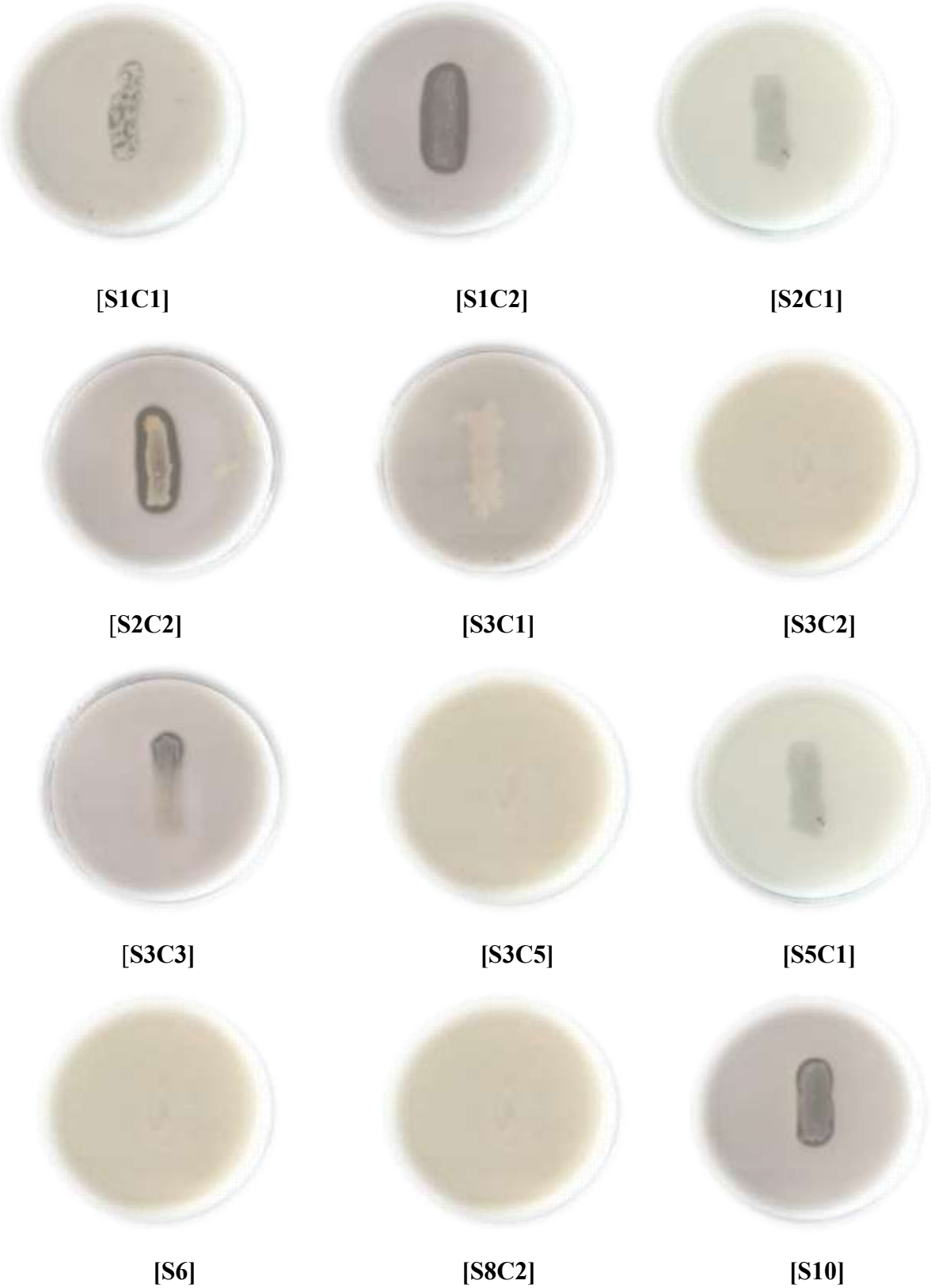

Figure 4 Acetic Acid Production Capacity on GYC Medium

Table 4 Acetic acid production in GYE media

Isolates	Acetic Acid Production (g/L)
S1C1	2.8
S1C2	5.0
S2C1	3.0
S2C2	5.4
S3	2.5
S4	2.7
S5	2.9
S6	3.1
S7	3.3
S10	4.2

3.5 Acetic Acid Tolerance Capacity

The acetic acid tolerance of isolates S1C2, S2C2, and S10 was assessed in GYE broth containing 1% to 5% v/v acetic acid over 7 days by measuring OD at 600 nm (Table 5, Table 6, Table 7; Figure 5, Figure 6, Figure 7). S1C2 showed good growth at 1% (0.86 OD) and 2% (0.82 OD) by day 7, moderate growth at 3% (0.48 OD), and minimal growth at higher concentrations (0.3–0.25 OD at 4% and 5%). S2C2 exhibited the highest acid tolerance, achieving 0.78 OD in 1%, 0.71 in 2%, 0.49 in 3%, and maintaining 0.32 OD even at 4% and 5% acetic acid, clearly outperforming the other isolates. In comparison, S10 showed moderate growth in 1% (0.6 OD) and 2% (0.51 OD), limited increase at 3% (0.3 OD), and negligible growth at 4% and 5%. These results confirm S2C2 as the most acid-tolerant isolate, capable of sustaining appreciable growth even under elevated acetic acid concentrations, making it a strong candidate for high-acidity industrial fermentation processes.

Table 5 S1C2 isolate Growth on GYE broth at different Acetic acid concentration

Days	OD of bacterial growth in 1% Acetic acid	OD of bacterial growth in 2% Acetic acid	OD of bacterial growth in 3% Acetic acid	OD of bacterial growth in 4% Acetic acid	OD of bacterial growth in 5% Acetic acid
0	0.3	0.3	0.3	0.3	0.3
1	0.39	0.38	0.3	0.3	0.3
2	0.45	0.4	0.32	0.3	0.3
3	0.51	0.48	0.36	0.3	0.3
4	0.58	0.55	0.4	0.32	0.28
5	0.65	0.63	0.42	0.35	0.27
6	0.77	0.74	0.45	0.39	0.25
7	0.86	0.82	0.48	0.43	0.25

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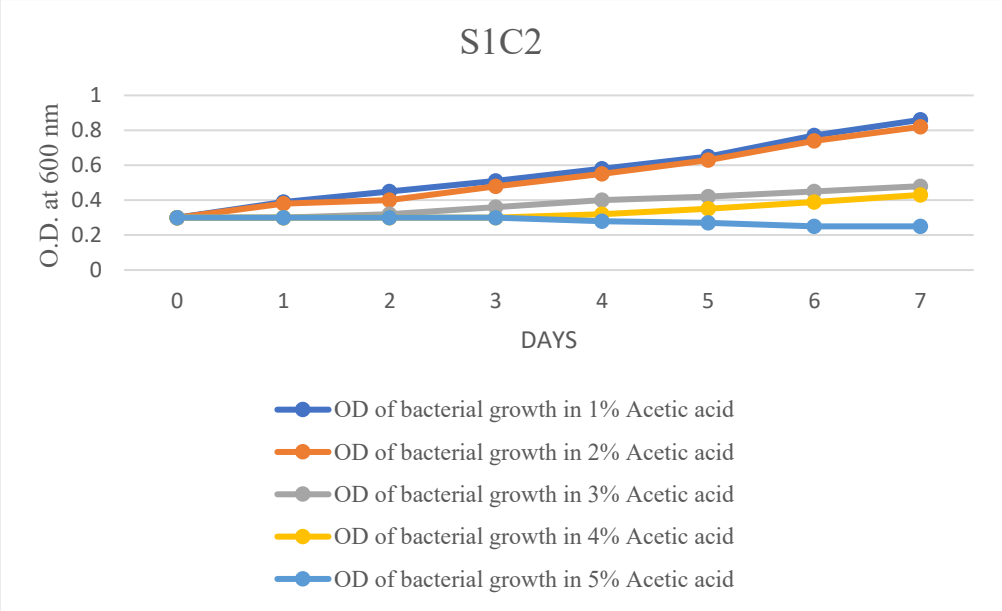

Figure 5 S1C2 Bacterial growth at different Acetic acid concentration

Table 6 S2C2 Bacterial growth at different Acetic acid concentration

Days	OD of bacterial growth in 1% Acetic acid	OD of bacterial growth in 2% Acetic acid	OD of bacterial growth in 3% Acetic acid	OD of bacterial growth in 4% Acetic acid	OD of bacterial growth in 5% Acetic acid
0	0.28	0.28	0.28	0.28	0.28
1	0.39	0.37	0.28	0.28	0.28
2	0.43	0.4	0.3	0.28	0.28
3	0.51	0.48	0.34	0.29	0.27
4	0.59	0.57	0.38	0.3	0.26
5	0.68	0.67	0.41	0.3	0.26
6	0.73	0.7	0.45	0.32	0.25
7	0.78	0.71	0.49	0.32	0.25

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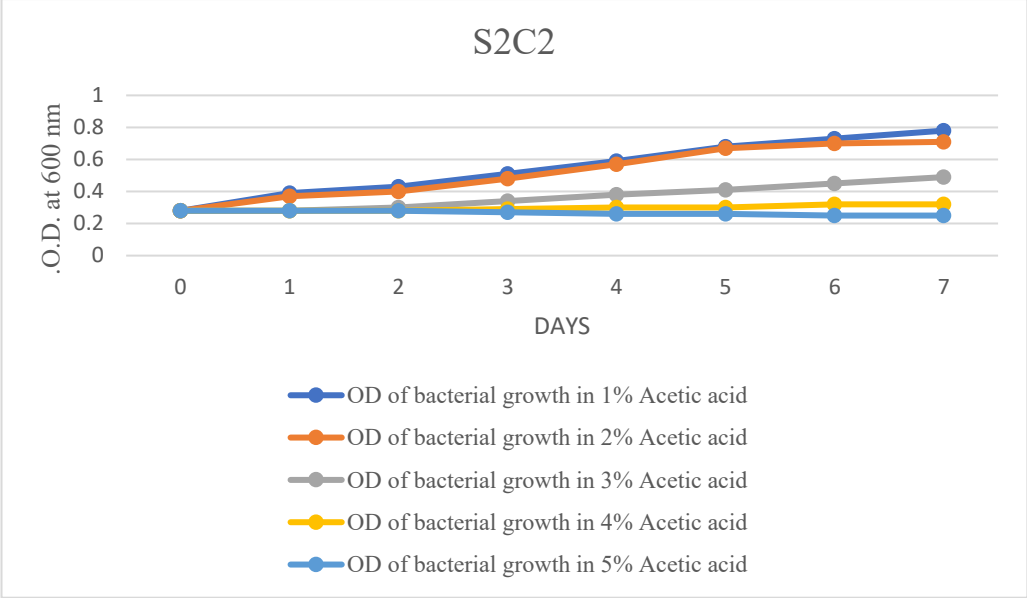

Figure 6 **S2C2 Bacterial growth at different Acetic acid concentration**

Table 7 S10 Bacterial growth at different Acetic acid concentration

Days	OD of bacterial growth in 1% Acetic acid	OD of bacterial growth in 2% Acetic acid	OD of bacterial growth in 3% Acetic acid	OD of bacterial growth in 4% Acetic acid	OD of bacterial growth in 5% Acetic acid
0	0.25	0.25	0.25	0.25	0.25
1	0.3	0.29	0.25	0.25	0.24
2	0.39	0.36	0.25	0.25	0.24
3	0.46	0.39	0.26	0.25	0.23
4	0.49	0.42	0.26	0.24	0.22
5	0.5	0.49	0.27	0.23	0.22
6	0.55	0.5	0.28	0.23	0.21
7	0.6	0.51	0.3	0.22	0.2

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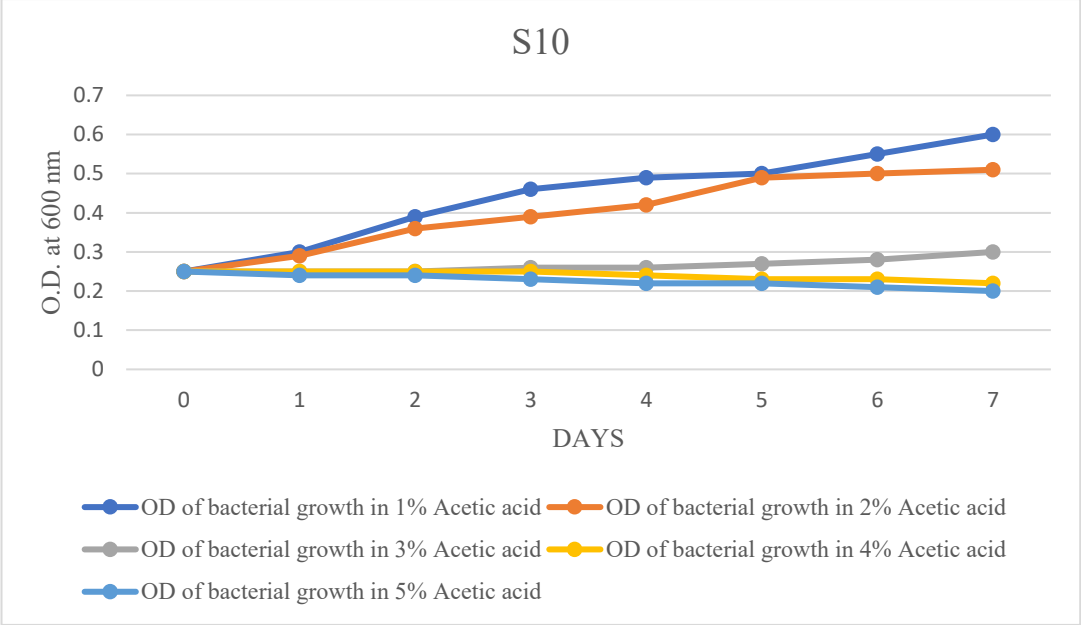

Figure 7 S10 Bacterial growth at different Acetic acid concentration

4. Discussion

The present study successfully isolated and screened ten bacterial strains from rhizosphere soils of diverse agricultural crops, confirming their potential as acetic acid producers. The preliminary screening using GYC agar demonstrated acidogenic potential in eight isolates through clear halo zones around colonies caused by $CaCO_3$ dissolution. Carr agar screening further validated acid production capacity, indicated by the color transition from purple to yellow due to pH changes around colonies. This rapid, qualitative identification technique has been widely employed for isolating AAB, corroborating methods described by Gullo et al. (2009) and reaffirmed in studies by Raspor and Goranovic (2008), who highlighted the effectiveness of $CaCO_3$-dissolving media for acid-producer selection.

Morphological characterization revealed colony consistency, pigmentation, and opacity consistent with classical descriptions of AAB genera such as Acetobacter and Komagataeibacter. Gram's staining confirmed that most acidogenic isolates were Gram-negative, short rods, matching findings by Illeghems et al. (2013), who similarly reported Gram-negative rod-shaped AAB dominant in vinegar fermentation systems. Notably, isolates S3C2, S5C1, S6, and S8C2 displayed Gram-positive reactions, indicating potential contamination or unrelated bacterial types, a common issue noted by De Roos and De Vuyst (2018), emphasizing the need for combined morphological and biochemical screening for accurate AAB identification.

Biochemical characterization of the three top-performing isolates (S1C2, S2C2, and S10) demonstrated consistent metabolic traits associated with AAB: all three tested positive for catalase and oxidase activities, confirming their obligate aerobic metabolism and reactive oxygen species defence mechanisms. Positive methyl red and citrate utilization tests further indicated their acidogenic and substrate versatility capabilities, comparable to results from Urbieta et al. (2017), who emphasized these traits in soil-derived AAB strains capable of surviving environmental stress and contributing to acetic acid fermentation. The absence of indole and VP reactions matched expected AAB profiles, reinforcing their tentative classification, as described similarly by Ozturk and Ercisli (2014) in studies on fruit-associated AAB.

Quantitative acetic acid production analysis revealed significant differences among isolates. S2C2 produced the highest acetic acid concentration (5.4 g/L) in GYE broth after 72 hours, outperforming S1C2 (5.0 g/L) and S10 (4.2 g/L). These values align closely with those reported by Gullo et al. (2009), who documented production ranges of 4.5–5.2 g/L for traditional Acetobacter strains, and are comparable to the 5.1 g/L observed by Coton et al. (2006) in food fermentation studies. The enhanced productivity of S2C2 highlights its industrial potential as an efficient, locally sourced vinegar fermentation strain.

Acetic acid tolerance assays confirmed the critical trait of acid resilience, essential for continuous vinegar production systems. S2C2 exhibited the highest tolerance, sustaining growth at 5% v/v acetic acid (0.32 OD by day 7), while S1C2 maintained moderate growth up to 4%, and S10 demonstrated limited tolerance beyond 3%. This observation is consistent with tolerance ranges reported by Illeghems et al. (2013) and Urbieta et al. (2017), who identified AAB strains tolerating 4%–6% v/v acetic acid in submerged cultures, and comparable to findings by Zhang et al. (2012) for Komagataeibacter strains resilient at 5%–6% acid concentrations.

Collectively, these findings confirm that rhizosphere soils serve as valuable ecological niches for metabolically versatile, acid-tolerant acetic acid bacteria. The combined morphological, biochemical, acid production, and tolerance profiles of isolate S2C2 demonstrate competitive and industrially desirable traits comparable to or exceeding those of standard AAB strains from fermented food sources. This supports the potential of indigenous soil-derived strains for local vinegar industries, offering adaptability to environmental conditions and possible cost reductions through reduced process controls.

5. Conflicts of Interest

Authors declare there is no conflicts of interest.

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