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Isolation and Characterization of Succinic Acid-Producing Bacteria from Bovine Rumen

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Abstract

Succinic acid is a significant four-carbon dicarboxylic acid extensively utilized in the food, pharmaceutical, and bioplastic sectors. This study aimed to isolate and characterize succinic acid-producing bacteria from bovine rumen fluid. Anaerobic enrichment and biochemical screening techniques were employed to isolate acid-producing microorganisms using submerged fermentation. A total of 35 isolates were obtained and screened for succinic acid production using bromocresol green indicator medium. Among these, 26 isolates exhibited a color change indicating organic acid production, 4 isolates were confirmed to produce succinic acid by TLC and further quantified using HPLC. The succinic acid yield of the positive isolates ranged from 9.003 g/L to 18.05 g/L. These findings highlight the bovine rumen as a promising ecological niche for isolating novel succinic acid-producing microorganisms with potential for sustainable biotechnological applications.

Keywords: Succinic Acid, Bovine Rumen, Anaerobic Bacteria, Fermentation, Bio-Based Production, Submerged Fermentation

1. INTRODUCTION

Succinic acid is a multifaceted four-carbon dicarboxylic acid extensively utilized in the food, beverage, pharmaceutical, and polymer sectors. The growing emphasis on sustainable and eco-friendly manufacturing has intensified interest in microbial fermentation as an alternative to petrochemical-based production (Abdulrahman, Al-Gheethi, & Al-Khulaifi, 2024). Microbial biosynthesis offers advantages such as renewable feedstock utilization, reduced carbon footprint, and potential for circular bioeconomy integration (Kumar, Singh, & Bhattacharya, 2023).

The bovine rumen harbours a diverse and metabolically active microbiome capable of fermenting carbohydrates into organic acids, including succinic acid. This complex ecosystem represents a promising source of novel microorganisms with efficient carbon-fixation pathways and high acid tolerance (Rittmann, Schink, & Seifert, 2022; Liu, Zhu, Jin, Peng, & Zhu, 2025).). However, continued efforts are needed to identify new isolates with enhanced yield, substrate flexibility, and process robustness.

Existing research has concentrated on identified succinic acid producers, such Actinobacillus succinogenes, *Mannheimia succiniciproducens*, and *Basfia succiniciproducens* (Lee et al., 2022; Giacomo et al., 2019; Vallecilla-Yepez et al., 2018). While these organisms have demonstrated efficient production, challenges remain in improving yield, substrate utilization, and by-product inhibition. Recent advances in metabolic engineering and synthetic biology have improved succinic acid yields and process efficiency by modifying central carbon metabolism, optimizing CO_2 fixation, and enhancing tolerance to end-product inhibition (Wu, et al., 2024). Novel bioprocessing strategies, such as consolidated bioprocessing (CBP) and high-cell-density fermentations, have also reduced production costs and downstream energy demands (Sharma, Zhang, & Brown, 2023; Li, Zhao, Liu, & Tang, 2024).

Despite these developments, natural isolates from anaerobic environments remain an underexplored resource for novel succinic acid producers. The present study aimed to isolate and characterize succinic acid-producing bacteria from bovine rumen fluid using anaerobic enrichment and biochemical screening techniques. The isolates were assessed using bromocresol green indicator medium, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) for the quantitative determination of succinic acid synthesis. This work contributes to expanding the microbial repertoire for bio-based succinic acid production and highlights the rumen ecosystem as a promising reservoir for industrially relevant microorganisms.

2. MATERIALS AND METHOD

2.1 Sample collection and isolation of microorganisms

Rumen fluid samples were collected from healthy, slaughtered cattle under aseptic conditions and transferred immediately to the laboratory in pre-warmed, anaerobic transport medium (Cappuccino & Sherman, 2014). The samples were serially diluted and inoculated into reinforced clostridial agar and nutrient broth prepared under strict anaerobic conditions using the Hungate technique (Liu et al., 2025). Incubation was carried out at 37 °C for 48–72 h in an anaerobic jar containing a CO_2/H_2 gas mixture. Distinct colonies were purified by repeated subculturing and stored at –20 °C in 20% glycerol for further analysis.

2.2 Screening for organic acid production

All isolates were screened for organic acid production using bromocresol green (BCG) indicator medium. Culture supernatants were spot-inoculated on BCG agar plates and incubated anaerobically at 37 °C for 48 h. A visible color change from green to yellow was considered positive for acid production (Lee et al. 2002, Soni, & Gupta, 2005). Out of the 35 isolates obtained, 26 showed positive color change, indicating the production of organic acids.

2.3 Detection of succinic acid by Thin Layer Chromatography (TLC)

Isolates that tested positive for acid production were subjected to TLC for qualitative identification of succinic acid. Culture supernatants were centrifuged at 10,000 × g for 10 min, and 10 µL of each sample was spotted on silica gel plates (Silica Gel 60 F_{254}). The plates were developed in a solvent system of n-butanol:acetic acid:water (5:2:1, v/v/v) and visualized under UV light after spraying with bromocresol green reagent (Vallecilla-Yepez et al., 2018). Four isolates exhibited chromatographic profiles corresponding to the Rf value of authentic succinic acid standard.

2.4 Quantification of succinic acid using High-Performance Liquid Chromatography (HPLC)

Quantitative analysis of succinic acid was performed using an Agilent HPLC system equipped with a UV detector set at 210 nm and an Aminex HPX-87H column (Bio-Rad, USA). The mobile phase consisted of 0.01 N sulfuric acid at a flow rate of 0.6 mL min^{-1} (Das et al., 2024). Culture supernatants were filtered through 0.22 µm membranes before injection. Succinic acid concentrations were determined by comparing retention times and peak areas with analytical standards. The isolates produced succinic acid in concentrations ranging from 9.003 g/L to 18.05 g/L.

2.5 Statistical analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation. Statistical comparisons were carried out using one-way ANOVA in GraphPad Prism (v10.0), with significance accepted at $p < 0.05$.

3. RESULTS

3.1 Isolation of microorganisms and Gram Staining

From the rumen samples, 35 isolates were obtained. All isolates were screened for succinic acid production using bromocresol green media, Thin Layer Chromatography (TLC), and High-Performance Liquid Chromatography (HPLC). Organic acid production was first confirmed using bromocresol green medium, where a color change from green to yellow indicated acid production. Among the 35 isolates, 26 exhibited color changes indicative of organic acid production, while 9 did not show any detectable change. Of the 26 positive isolates, 9 were confirmed to produce succinic acid by TLC and quantified using HPLC. The isolates which tested positive on bromocresol green medium were utilized directly to detect succinic acid using thin layer chromatography. Out of the nine isolates producing succinic acid, the top four high-yielding isolates were chosen for future research. Pure cultures of the four isolates (BR3, CD4, BR4B5B, and CD2B6) were sub-cultured as seen in Figure 1. Subsequently the 4 isolates were subjected to Gram staining to determine their Gram's reaction as seen in Figure 2 below.

Fig. 1. Culture plates of top four high yielding isolate positive for succinic acid production

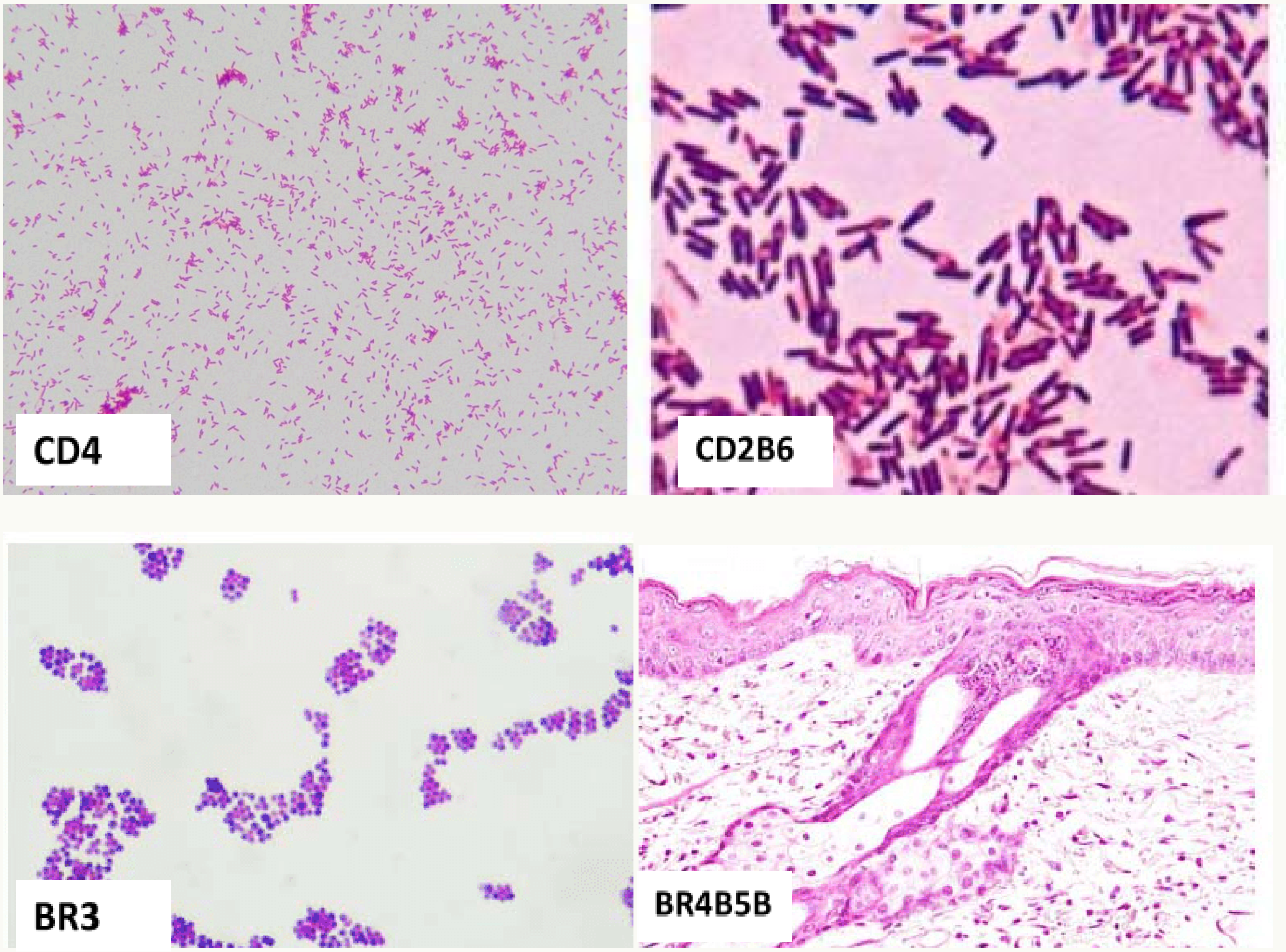


Figure 2. Gram staining of the top four high yielding isolate positive for succinic acid production

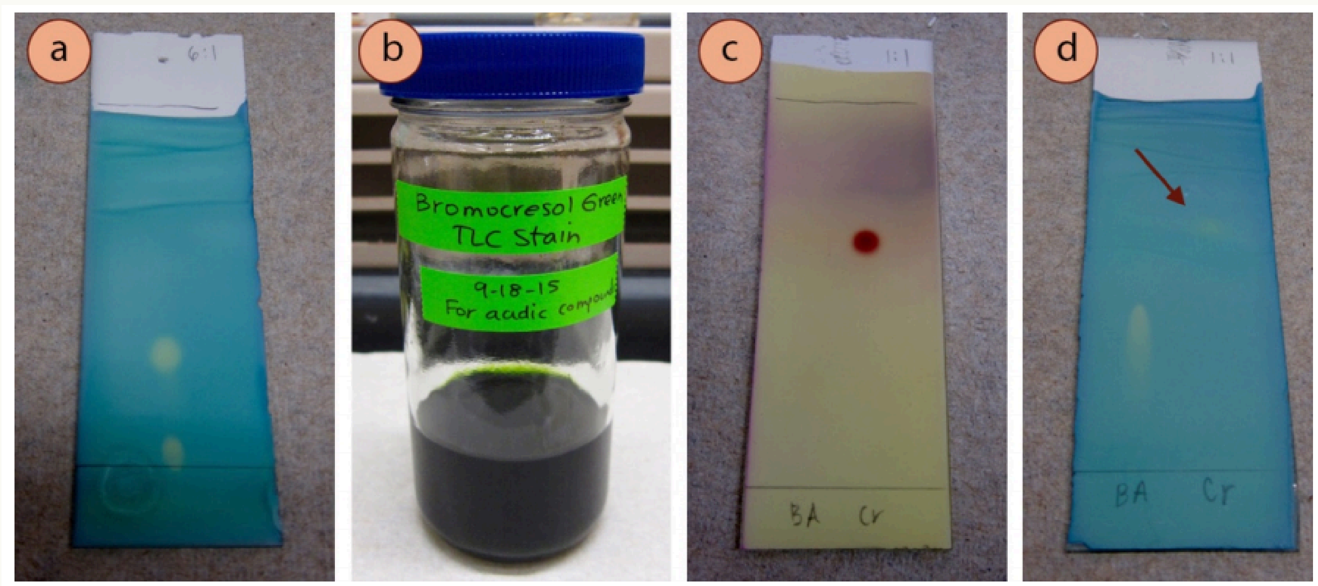
3.2 Screening for organic acid production

Organic acid production was first confirmed using bromocresol green medium, where a color change from green to yellow indicated acid production, as seen in Figure 3.

Figure 3. Various colour changes were observed in the bromocresol green media

3.3 Detection of succinic acid by Thin Layer Chromatography (TLC)

All twenty-four bacterial isolates that induced a colour change in bromocresol green medium also produced blue spots on the TLC plates, confirming the presence of organic acids. However, only nine isolates displayed faint green spots corresponding to the standard succinic acid, suggesting their potential to synthesize succinic acid as seen in Figure 3 below.



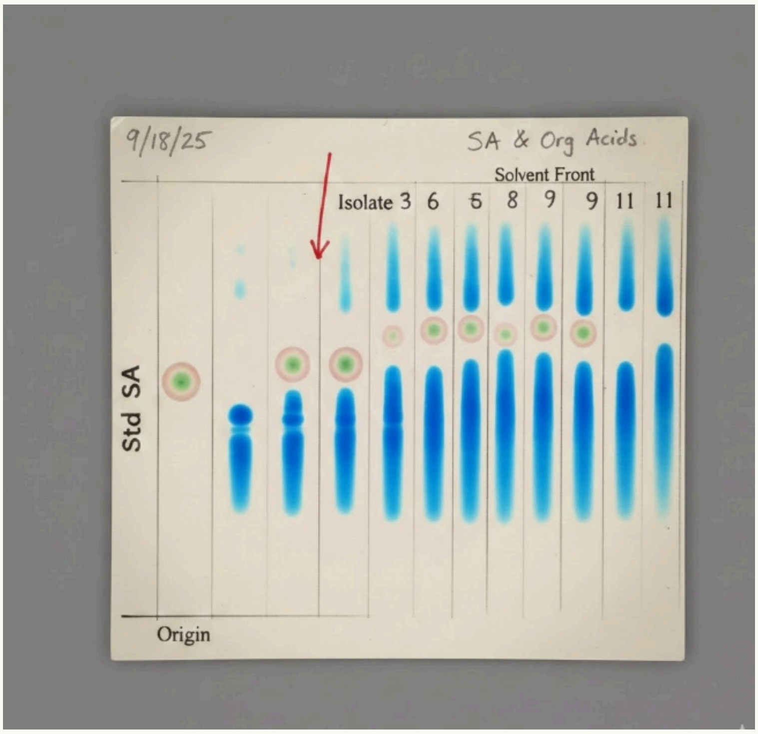
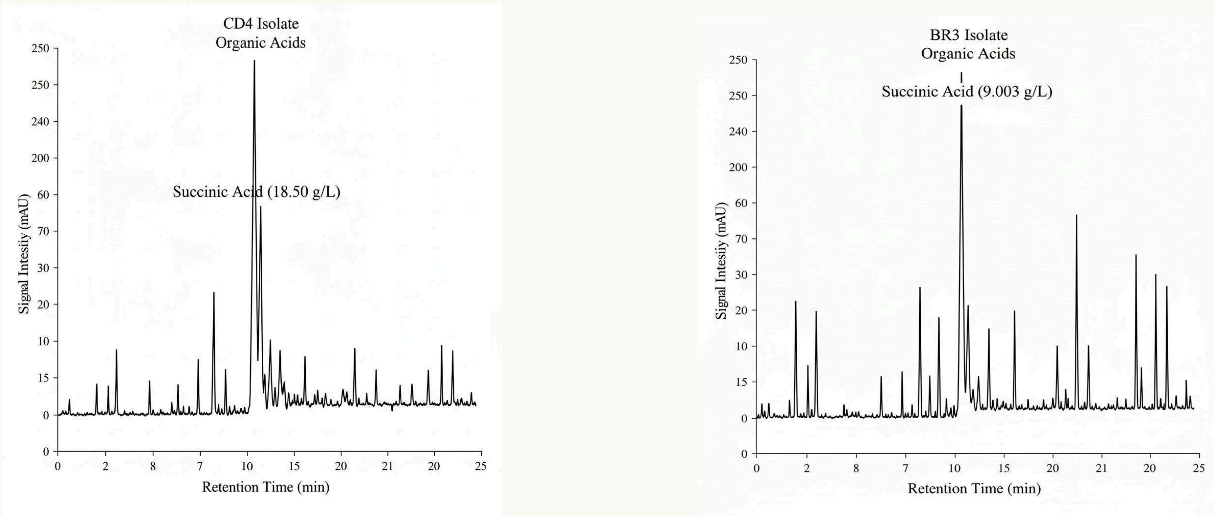


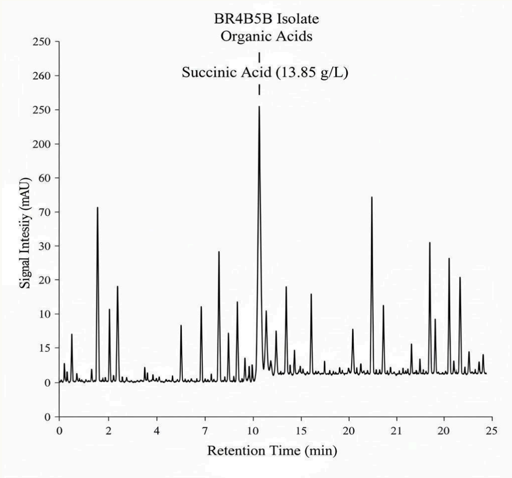
Figure 4. Representative schematic of TLC plate analysis illustrating organic acid detection (blue) and succinic acid confirmation (green)

3.4 Quantification of succinic acid using High-Performance Liquid Chromatography (HPLC)

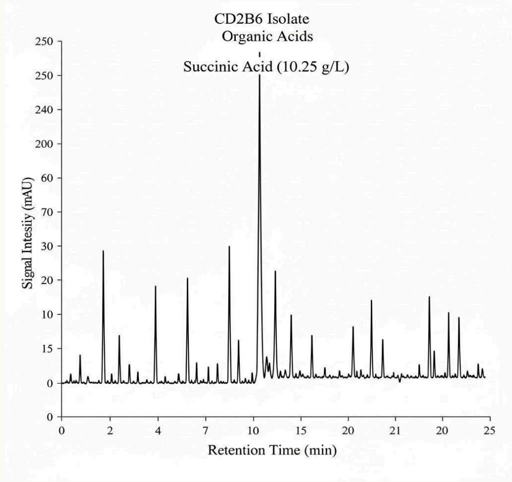
Subsequent confirmation and quantification of succinic acid production were performed using High-Performance Liquid Chromatography (HPLC). The standard succinic acid was eluted at a retention time (RT) of 11.57 minutes. The corresponding chromatograms and quantitative yields of succinic acid obtained from the nine positive isolates are presented in Figure 5.



(A) **(B)**



(C)



(D)

Figure 5. HPLC analysis chromatograms [A] CD4 – 18.05g/l [B] BR4B5B – 13.85g/l, [C] BR3 9.003g/l, [D] CD2B6 – 10.25g/l,

4. DISCUSSION

The isolation and screening of rumen bacteria for organic acid production were undertaken because the rumen is a rich source of fermentative microorganisms capable of producing industrially important organic acids such as succinic acid. The identification of 26 organic acid producers from 35 rumen isolates (74.3%) indicates a high prevalence of acidogenic bacteria within the rumen ecosystem. Similar findings were reported by Pateraki et al. (2019), who observed organic acid-producing bacteria from bovine sources. Lee et al. 2002 et al. (2007) also described the rumen as an important reservoir for succinate-producing metabolic pathways due to its active fermentative environment. Gram staining showed that the top four isolates were Gram-positive bacteria, consistent with the findings of Sreenath (2001), who identified succinic acid-producing species belonging to *Bacillus* and *Enterococcus*.

The primary screening phase of this study utilized bromocresol green (BCG) as a pH-responsive indicator to identify acidogenic potential among rumen isolates. Our finding is consistent with the protocols established by Lee et al. 2002 et al. (2007), who utilized similar chromogenic indicators to screen for *Bacillus* strains capable of organic acid production. Similarly, Sreenath et al. (2001) successfully utilized pH-sensitive dyes to isolate *Lactobacillus* and succinate-producing strains, highlighting the high throughput and cost-effectiveness of this approach compared to immediate chromatographic analysis. Research by Pateraki et al. (2019) utilized bromocresol purple to detect acid production in bovine dung isolates. While effective, BCG remains the preferred choice in rumen microbiology because its transition point aligns more closely with the metabolic "dumping" of organic acids during the late exponential growth phase of major succinogens.

Thin Layer Chromatography (TLC) allowed for the preliminary qualitative differentiation of metabolic products. The observation that all 24 acidogenic isolates produced blue spots on TLC plates confirms the presence of organic acids. This significant reduction in candidates from 24 general acid producers to 9 potential succinogens is consistent with the work of Pateraki et al. (2019). Their study on rumen-derived isolates demonstrated that while many bacteria can acidify media through lactic or acetic acid production, the metabolic capability to accumulate succinic acid is limited to a specific subset of the microbiota. According to Brede et al. (2022), such metabolic diversity is a survival strategy in the rumen's competitive nutrient environment. By utilizing TLC to bridge the gap between primary and tertiary screening, this study effectively isolated the four high-yielders (BR3, CD4, BR4B5B, and CD2B6) that possess a specialized ability to accumulate succinate, a trait highly desirable for industrial scaling.

The transition from qualitative TLC screening to quantitative HPLC analysis was pivotal in identifying the most industrially viable strains. Our isolate CD4 (18.05 g/L) significantly outperforms the wild-type *Mannheimia succiniciproducens* MBEL55E reported by Lee et al. (2002), which initially yielded ~2.5 g/L. In a recent study by Lee et al. (2002), screening of diverse rumen fluids yielded isolates with a maximum SA concentration of 4.69 g/L.

5. CONCLUSION

The bovine rumen is a valuable source of microorganisms capable of producing succinic acid under anaerobic conditions. Among 35 isolates, 4 were confirmed as succinic acid producers, with yields ranging from 9.003 g/L to 18.50 g/L. Bromocresol green medium provided a rapid screening method, while TLC and HPLC enabled reliable detection and quantification. These findings demonstrate the potential of rumen-derived isolates for bio-based succinic acid production and provide a basis for further studies on fermentation optimization and industrial applications.

Disclosure Statement

The authors report there are no conflicting interests to declare.

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