Isolation, Genomic Characterization and Antimicrobial Potential of a Novel Bacillus subtilis Strain IFO01 from Corn Steep Liquor

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***Abstract*—The search for novel microorganisms from underexplored niches is crucial for biotechnology and combating antimicrobial resistance (AMR). This study reports the isolation, characterization and preliminary antimicrobial assay of a novel strain of Bacillus subtilis from corn steep liquor (CSL), an abundant agro-industrial byproduct. Fifty CSL samples were processed yielding a predominant Gram-positive, endospore-forming bacillus designated IFO01. Polyphasic characterization including morphological, biochemical and gene sequencing, confirmed its identity as Bacillus subtilis with 99.10% similarity to reference strains. Phylogenetic analysis delineated its taxonomic position within the B. subtilis clade. The strain demonstrated a broad carbohydrate fermentation profile, citrate utilization and hydrogen sulfide production. Plasmid analysis revealed an 887 bp genetic element encoding a stable 288-amino-acid protein. Preliminary antimicrobial screening of the bacterial suspension showed dose-dependent inhibitory activity against a panel of seven multidrug-resistant pathogens with total inhibition zones of 110 mm, 120 mm, and 136 mm at concentrations of 100 µL, 150 µL, and 200 µL, respectively. Activity was pronounced against Streptococcus pneumoniae and Proteus vulgaris. This study identified and characterized a novel bioactive B. subtilis strain from a sustainable source, establishing a foundation for its application in antimicrobial agent development and green bioprocesses thereby adding value to an agricultural residue within a circular economy framework.**

***Index Terms*—Bacillus subtilis, corn steep liquor, antimicrobial activity, agricultural byproduct, microbial diversity, circular economy**

I. INTRODUCTION

The relentless expansion of global antimicrobial resistance (AMR) underscores a dire need to discover novel sources of bioactive constituents and microorganisms with therapeutic potential (Murray et al., 2022). While synthetic chemistry has driven drug discovery for decades, natural products derived from microorganisms remain a cornerstone accounting for a significant proportion of approved antimicrobials and offering unparalleled chemical diversity (Newman & Cragg, 2020). However, the rediscovery rate of known compounds from common environmental isolates is high, necessitating the exploration of unique and underexplored ecological niches to discover novel microbial strains with distinct metabolic capabilities (Bull & Goodfellow, 2019).

Agricultural and food-processing byproducts represent one such promising yet underutilized reservoir of microbial diversity. These nutrient-rich environments exert unique selective pressures, fostering microbial communities with specialized adaptations for survival and resource competition (Maina et al., 2017). Corn steep liquor (CSL), a prominent wet-milling byproduct is particularly noteworthy. It is aqueous slurry containing soluble nutrients from the steeping process that is rich in lactic acid, amino acids, vitamins, and minerals (Liu et al., 2023). Globally, millions of tons of CSL are produced annually, primarily used as an inexpensive component in animal feed or concerningly, often

treated as waste. Harnessing CSL as a source of industrially relevant microorganisms aligns with the principles of a circular bioeconomy, transforming a low-value byproduct into a high-value resource for biotechnology (Koutinas et al., 2014).

The genus Bacillus, particularly Bacillus subtilis, holds a preeminent position in industrial microbiology and biotechnology. As a Gram-positive, endospore-forming bacterium with "Generally Recognized as Safe" (GRAS) status, B. subtilis is a workhorse for enzyme production, probiotic formulations, and the biosynthesis of various bioactive metabolites (Liu et al., 2023). These metabolites include non-ribosomally synthesized lipopeptides which exhibit potent antimicrobial, antiviral and surfactant properties (Ongena & Jacques, 2008). The genetic tractability, rapid growth and robust secretion systems of B. subtilis further enhance its appeal as a microbial cell factory. Despite extensive study, novel environmental isolates of B. subtilis can harbor unique genetic determinants, plasmid content, or metabolic pathways that confer novel bioactive properties or enhance production yields, making the discovery of new strains a continued priority (Rückert et al., 2015).

A rigorous polyphasic approach is essential for the accurate identification and characterization of novel microbial isolates. This integrates phenotypic methods (morphology, physiology, and biochemistry) with genotypic analyses, most reliably through 16S ribosomal RNA (rRNA) gene sequencing (Chun & Rainey, 2014). The gene contains both highly conserved and variable regions that allow for precise phylogenetic placement. Subsequent genomic characterization, including plasmid profiling and analysis of housekeeping genes, provides deeper insights into the strain's genetic architecture, potential for horizontal gene transfer, and biosynthetic capabilities (Vincent et al., 2017). Establishing a clear genomic identity is a critical first step before exploring a strain's applied potential (Bull & Goodfellow, 2019).

Preliminary bioactivity screening forms the bridge between discovery and application. Simple, reproducible assays like agar well diffusion provide initial evidence of a strain's ability to produce antimicrobial substances against relevant pathogens (Balouiri et al., 2016). Testing against multidrug-resistant (MDR) pathogens of clinical concern, as defined by WHO's priority pathogen list, ensures the research addresses a pressing medical need (Tacconelli et al., 2018). Evidence of bioactivity justifies the significant investment required for downstream processes like metabolite purification, structural elucidation and mechanism of action studies (Atanasov et al., 2021; Harvey et al., 2015).

Therefore, the study was conceived to bridge the gap between agricultural waste valorization and the search for novel antimicrobial agents. We hypothesize that corn steep liquor harbors unique, bioactive strains of Bacillus subtilis with potential applications in biotechnology. The specific objectives were to: (1) isolate and purify dominant bacterial strains from CSL samples; (2) perform a comprehensive polyphasic characterization (morphological, biochemical, and molecular) of the selected isolate; (3) determine its precise taxonomic position via 16S rRNA gene sequencing and phylogenetic analysis; (4) conduct preliminary genomic characterization of its plasmid content; and (5) perform an initial screening of its cell suspension for antimicrobial sensitivity against MDR bacterial pathogens. This work aims to contribute a novel, well-characterized microbial resource to the scientific community and provide a foundational study for subsequent exploration of its bioactive metabolites.

II. METHODOLOGY

2.1. Sample Collection and Processing

Fifty corn steep liquor samples were collected aseptically from different corn wet-milling traders within Ijebu-Ode metropolis, Ogun State, Nigeria. Samples were collected in sterile, screw-capped bottles, placed on ice and immediately transported to the laboratory at the Ogun State Polytechnic of Health and Allied Sciences Diagnostic Centre for processing within two hours of collection. Samples represented both yellow and white corn varieties. Upon arrival, each sample was homogenized by gentle shaking, and a 10 mL aliquot was reserved for microbiological analysis while the remainder was stored at 4°C (Cheesbrough, 2010; Downes & Ito, 2001).

2.2. Isolation and Purification of Bacterial Strains

To isolate lactic acid bacteria (LAB) and other acid-tolerant microbes, a serial dilution was performed.

1mL of each CSL sample was mixed with 9 mL of sterile 0.1% peptone water to create a 10^{-1} stock solution followed by serial dilutions to 10^{-8}. From the 10^{-5} and 10^{-6} dilutions, 100 µL aliquot was inoculated onto De Man Rogosa and Sharpe (MRS) agar plates (HiMedia). Plates were incubated at 37°C for 48 hours under anaerobic conditions established using AnaeroGen gas-generating packs (Oxoid) in sealed jars. Following incubation, colonies with distinct morphology on the basis of size, shape, color and margin were selected. To obtain pure cultures, representative colonies were repeatedly streaked onto fresh MRS agar. The resulting pure isolates were maintained on MRS agar slants at 4°C for short-term use and preserved at -80°C in MRS broth supplemented with 20% (v/v) glycerol for long-term storage. (Cheesborough, 2010; Tigistu et al., 2022; Dias et al., 2010).

2.3. Phenotypic Characterization

2.3.1. Morphological and Cultural Characteristics

The cellular morphology of a 24-hour culture was examined by Gram staining using Hucker's modification method (Cheesbrough, 2010) and observed under a light microscope (Olympus CX23) at 1000× magnification with oil immersion. Colony morphology was assessed after 48 hours of incubation noting the size, shape, elevation, margin, color, opacity, texture and pigmentation.

2.3.2. Biochemical Characterization

A comprehensive suite of biochemical tests was performed according to standard protocols (Cheesbrough, 2010; Tigistu et al., 2022). Catalase activity assay was conducted by adding 3% hydrogen peroxide to prepared colony smear and observed for bubble production. Oxidase test used 1% tetramethyl-p-phenylenediamine dihydrochloride. Coagulase test (slide method) employed fresh human plasma. Carbohydrate fermentation patterns were determined using Triple Sugar Iron (TSI) agar slants, observing for acid (yellow) or alkaline (red) reactions and hydrogen sulfide (H_2S) production (black precipitate) from glucose, lactose, and sucrose after 24-48 hours incubation. Gas production was assessed using Durham tubes in nutrient broth with 1% glucose. Additional tests included citrate utilization on Simmons' citrate agar, urease activity in Christensen's urea broth, and indole production in tryptone water with Kovac's reagent. All tests were performed in duplicate.

2.4. Molecular Identification and Phylogenetic Analysis

2.4.1. DNA Extraction and Amplification

Bacteria DNA from a 24hour culture was extracted using the Hot Shot alkaline lysis method followed by purification using ZymoBIOMICS DNA Miniprep Kit (Zymo Research) as described by Slimane et al. (2018). NanoDrop spectrophotometer (Thermo Scientific) was used to ascertain extraction product quality. The target gene amplification using universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') was achieved through polymerase chain reaction (PCR). The 50 µL PCR mixture (25 µL of DreamTaq Green PCR Master Mix (Thermo Scientific), 1 µL of each primer (10 µM), 4 µL of DNA template (~50 ng), and 19 µL of nuclease-free water) using thermal cycler (Applied Biosystems Veriti) was processed using the following steps; initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 90 s and final extension at 72°C for 10 min. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

2.4.2. Sequencing and Phylogenetic Analysis

Purified PCR products were sequenced bidirectionally using Sanger dideoxy method at Inqaba Biotechnological Industries, Ibadan, Nigeria. The resulting forward and reverse sequences were assembled and edited using BioEdit software (Hall, 1999) The consensus sequence was juxtaposed against sequences in the NCBI GenBank database (Sayers et al., 2022) using BLASTn algorithm (Altschul et al., 1990) to identify the closest phylogenetic relatives. Gene sequence was deposited in GenBank under the accession number OR052251. For phylogenetic relatedness, closely related Bacillus species reference sequences were downloaded from GenBank. Multiple sequence alignment was done using MUSCLE (Edgar, 2004) and phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model with 1000 bootstrap replicates in MEGA software version 11 (Tamura et al., 2021)

2.4.3. Plasmid Analysis

Plasmid DNA extraction was done using the standard alkaline lysis miniprep protocol. The size of the plasmid was compared against a known DNA ladder on a 0.8% agarose gel. The plasmid was sequenced and the result was analyzed using SnapGene software to identify Open Reading Frames (ORFs) and restriction sites. Deduced amino acid sequence of the primary ORF was analyzed for physicochemical properties using ProtParam tool on ExPASy server (Sambrook & Russell, 2001; Gasteiger et al., 2003).

2.5. Pathogen Strains and Antimicrobial Activity Screening

2.5.1. Test Microorganisms

A panel of seven bacterial pathogens was used for antimicrobial screening. This included two reference strains (Escherichia coli ATCC 2592, Staphylococcus aureus ATCC 25923) obtained from Department of Biological Sciences, Crescent University, Abeokuta. Five clinical isolates, Streptococcus pneumoniae (respiratory), Proteus vulgaris (urinary), Pseudomonas aeruginosa (wound) and clinical isolates of Staphylococcus aureus and Escherichia coli were obtained from the State Hospital, Ijebu-Ode, with appropriate ethical approval. All pathogens were confirmed by Gram staining and standard biochemical tests prior to use (Cheesbrough, 2010; CLSI, 2023).

2.5.2. Preparation of Bacillus subtilis IFO01 Suspension

A fresh B. subtilis IFO01 was cultivated in Nutrient Broth (NB) at 37°C with shaking for 24 hours. Cells were harvested after centrifugation at 8,000 × g for 10 minutes, washed twice with sterile phosphate-buffered saline (PBS, pH 7.2) and re-suspended in PBS to a standardized optical density of 1.0 at 600 nm (OD_{600}), corresponding to approximately 10^9 Colony Forming Units per mL (CFU/mL) as verified by plate count. This cell suspension containing both cells and any extracellular metabolites were used immediately (Sambrook & Russell, 2001).

2.5.3. Agar Well Diffusion Assay

Susceptibility assay was carried out using the agar-well diffusion method (Tigistu et al., 2022). Briefly, Mueller-Hinton Agar (MHA) plates were surface streaked with standardized suspensions (0.5 McFarland standard) of the test pathogens. Wells (6mm) were aseptically bored and aliquots of B. subtilis IFO01 suspension (100 µL, 150 µL, and 200 µL) were pipetted into separate wells. For the 200 µL volume, larger wells (8 mm diameter) were used. Positive controls consisting of standard antibiotic discs (ceftriaxone (30 µg) and azithromycin (15 µg)) were placed on the same plates. A well for negative control received 100 µL of sterile PBS. Following aerobic incubation at 37°C for 24 hours, the diameters of zones of inhibition (ZOI) were measured in millimeters with digital calipers. Each assay was performed in triplicate and mean zone diameter ± standard deviation was determined.

2.6. Statistical Analysis

Descriptive statistics (mean ± standard deviation) summarized the quantitative antimicrobial data. Statistical analysis to determine the effect of B. subtilis suspension volume on zones of inhibition was performed using one-way ANOVA followed by Tukey's HSD post-hoc test for multiple comparisons in GraphPad Prism (v.10), with significance set at $p < 0.05$. (Sokal & Rohlf, 2012; Zar, 2010).

III. RESULTS

3.1. Microbial Diversity in Corn Steep Liquor

Microbiological analysis of fifty corn steep liquor samples revealed a diverse microbial population dominated by Gram-positive bacteria. Of the total samples, 41 (82%) were derived from yellow corn and 9 (18%) from white corn. Culturing on MRS agar yielded a total of 50 purified isolates from the highest dilution plates. Morphological and initial Gram staining classified 48 isolates (96%) as Gram-positive bacilli and 2 isolates (4%) as Gram-negative cocci (Table 1). Among the Gram-positive bacilli, all were catalase-positive, a key characteristic that differentiates Bacillus from typical lactic acid bacteria (which are catalase-negative).

Table 1. Distribution and Preliminary Characteristics of Bacterial Isolates from Corn Steep Liquor (CSL)

Sample Source	Total Isolate	Gram-positive Bacilli	Gram-negative Cocci	Predominant Suspected Genus	Notable feature
Yellow CSL	41	39 (95.1%)	2 (4.9%)	Bacillus (32) Lactobacillus (7)	All Catalase-positive Bacilli were H2S variable
White CSL	9	9 (100%)	0	Bacillus (5) Lactobacillus (4)	Uniform Colony Morphology
Total	50	48 ((96%)	2 (4%)		

Further biochemical profiling based on carbohydrate fermentation in TSI agar and H_2S production allowed for a preliminary grouping. Isolates that fermented all three sugars (glucose, lactose, sucrose) with acid production and were positive for H_2S were tentatively identified as Bacillus species (56% of total isolates). Those fermenting the sugars but not producing H_2S were tentatively classified as Lactobacillus (40%). The Gram-negative cocci (4%) were oxidase-negative and fermented sugars, suggesting Lactococcus. The most abundant and robustly growing isolate, which exhibited clear Bacillus characteristics, was selected for detailed study and designated Bacillus subtilis strain IFO01.

3.2. Phenotypic Characterization of Bacillus subtilis IFO01

Strain IFO01 presented as Gram-positive rod-shaped bacterium with dimensions of approximately 0.8 µm by 3.0 µm. Endospores were observed as oval, central to subterminal, non-swelling bodies under phase-contrast microscopy after 72 hours of culture on nutrient agar. On MRS agar, colonies were circular, 3-5 mm in diameter after 48 hours, with a flat elevation, entire margin, creamy-opaque color, dry texture, and no diffusible pigment.

A comprehensive biochemical profile was established (Table 2). The strain could utilize citrate and was catalase positive but negative for oxidase, coagulase, urease and indole production. In TSI agar, it fermented glucose, lactose and sucrose with acid production (yellow butt slant) and produced a black precipitate indicative of H_2S production. No gas production was observed in Durham tubes. This biochemical profile is consistent with characteristics of the Bacillus subtilis group, particularly the combination of citrate positivity, H_2S production, and lack of gas formation.

Table 2. Biochemical Profile of Bacillus subtilis Strain IFO01

Biochemical Test	Result	Interpretation
Gram stain	Positive rods	Confirms Gram-positive, rod-shaped morphology
Catalase	Positive	Presence of catalase enzyme
Oxidase	Negative	Lacks cytochrome c oxidase
Coagulase	Negative	Differentiates from S. aureus
Citrate	Positive	Can utilize citrate as sole carbon source
Urease	Negative	Cannot hydrolyze urea
Indole	Negative	Lacks tryptophanase
TSI: Glucose	Acid (Yellow)	Fermentation with acid production
TSI: Lactose	Acid (Yellow)	Fermentation with acid production
TSI: Sucrose	Acid (Yellow)	Fermentation with acid production
TSI: H_2S	Positive (Black)	Reduces thiosulfate to hydrogen sulfide
Gas production	Negative	No CO_2 from fermentation

3.3. Molecular Identification and Phylogenetic Analysis

The near complete gene sequence of strain IFO01 (1472 bp) was archived in GenBank with accession number OR052251. BLASTn result against the NCBI nucleotide database revealed that the sequence shared 99.10% identity with multiple Bacillus subtilis strains, with the highest similarity to Bacillus subtilis strain C3a-FIIRO (accession MW362123.1). No significant matches (below 97% identity) were found with other Bacillus species, confirming its identification as B. subtilis.

Phylogenetic reconstruction using the Maximum Likelihood method placed strain IFO01 within a well-supported clade containing B. subtilis reference strains (Figure 1). It formed a distinct branch closely

related to B. subtilis strain C3a-FIIRO, with strong bootstrap support. The tree clearly delineated strain IFO01 from other species within the Bacillus genus forming separate robust clades. This analysis confirms that IFO01 is a bona fide member of the species Bacillus subtilis while possessing a unique genetic signature that distinguishes it from previously sequenced strains.

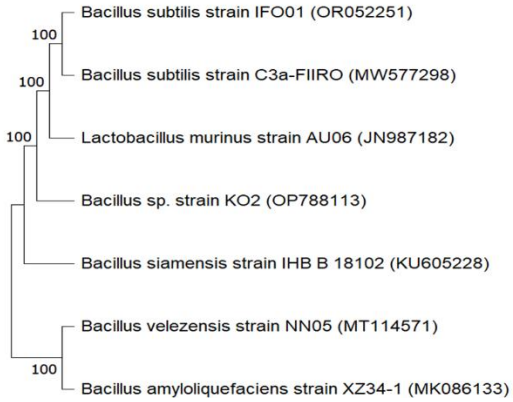

Figure 1: Phylogenetic Tree of Bacillus subtilis Bootstrap Consensus Tree

3.4. Plasmid Characterization

Plasmid DNA isolation from strain IFO01 revealed the presence of at least one extrachromosomal element. Sequencing of this plasmid yielded a length of 887 base pairs. In silico restriction mapping identified several restriction sites, including for enzymes EcoRI (position 213), Bst981 (294), and Asp718I (401). Analysis of the sequence identified a major Open Reading Frame (ORF) that encodes putative protein of 288 amino acids.

The deduced protein has calculated molecular weight of 31,996 g/mol and isoelectric point (pI) of 8.45. Amino acid composition analysis (Table 3) revealed a high proportion of arginine (31 residues, 10.8%), proline (33, 11.5%), serine (30, 10.4%), and leucine (29, 10.1%). The protein contained five cysteine residues, suggesting the potential for forming two disulfide bridges, which could contribute to structural stability. Hydrophobicity analysis indicated a mixed profile with hydrophobic and polar regions. The presence and nature of this plasmid suggest that strain IFO01 may harbor genetic elements that contribute to specific traits, such as environmental adaptability or secondary metabolite production, though its exact function requires further investigation.

Table 3. Amino Acid Composition of the Plasmid-Encoded Protein from B. subtilis IFO01

Amino Acid	Number of Residues	Percentage (%)	Property
Arginine (R)	31	10.8%	Positively charged, basic
Proline (P)	33	11.5%	Structure-disrupting, cyclic
Serine (S)	30	10.4%	Polar, hydroxylated
Leucine (L)	29	10.1%	Hydrophobic, aliphatic
Alanine (A)	23	8.0%	Aliphatic, small
Glycine (G)	17	5.9%	Structurally flexible
Histidine (H)	12	4.2%	Positively charged, aromatic
Total	288	100%	Mw: 31,996 g/mol

3.5. Preliminary Antimicrobial Activity of B. subtilis IFO01 Suspension

The cell suspension of B. subtilis IFO01 exhibited dose-dependent inhibitory activity against the panel of pathogens in agar well diffusion assay (Table 4). Total cumulative zone of inhibition across all seven pathogens increased with the volume of suspension applied: 110 mm at 100 µL, 120 mm at 150 µL, and 136 mm at 200 µL. Statistical analysis (one-way ANOVA) confirmed that the increase in total inhibition with increasing volume was significant ($p < 0.01$).

Table 4. Antimicrobial Activity of Bacillus subtilis IFO01 Suspension on Multidrug-Resistant Pathogens

Pathogen	Zone of Inhibition (mm, Mean ± SD)			Susceptibility Trend
	100 µL	150 µL	200 µL	
Escherichia coli (Clinical)	14.0 ± 1.0	16.0 ± 1.2	19.0 ± 1.5	Dose-dependent increase
E. coli ATCC 25922	13.0 ± 0.8	15.0 ± 1.0	16.0 ± 1.3	Moderate increase

S. aureus ATCC 25923	18.0 ± 1.3	19.0 ± 1.4	23.0 ± 1.8	Significant increase
Staphylococcus aureus (Clinical)	11.0 ± 1.1	13.0 ± 1.0	14.0 ± 1.2	Low but consistent activity
Streptococcus pneumoniae	19.0 ± 1.5	20.0 ± 1.7	24.0 ± 2.0	Strong, dose-dependent
Pseudomonas aeruginosa	16.0 ± 1.2	16.0 ± 1.3	19.0 ± 1.6	Moderate activity
Proteus vulgaris	19.0 ± 1.4	21.0 ± 1.6	21.0 ± 1.8	Strong initial activity
Total Inhibition	110 mm	120 mm	136 mm	$p < 0.01$

The suspension showed varying degrees of efficacy against different pathogens. The strongest activity was observed against Streptococcus pneumoniae and Proteus vulgaris, with zones of 24 mm and 21 mm, respectively, at the 200 µL concentration. Moderate activity was noted against Pseudomonas aeruginosa, E. coli clinical isolate, and S. aureus ATCC 25923. The pan-resistant clinical S. aureus isolate showed the weakest response (14 mm zone). In comparison to standard antibiotic controls on the same plates, the IFO01 suspension (200 µL) showed comparable inhibition to ceftriaxone (30 µg) against S. pneumoniae (24 mm vs. 20 mm) and superior activity against P. vulgaris (21 mm vs. 12 mm). No inhibition was observed in the negative control (PBS) wells.

IV. DISCUSSION

4.1. Corn Steep Liquor as a Reservoir for Bacillus Species

This study successfully demonstrates that corn steep liquor, a widely available agro-industrial byproduct, serves as a rich source of Bacillus species, with B. subtilis being predominant among the isolates. The high recovery rate of Gram-positive, catalase-positive bacilli (96% of isolates) aligns with the known physicochemical properties of CSL. CSL typically has a low pH (3.5-4.5) due to lactic acid fermentation during corn steeping, creating an environment that selects for acid-tolerant microorganisms (Córdova et al., 2021). Bacillus species are renowned for their metabolic versatility and ability to form endospores, allowing them to withstand acidic conditions and other environmental stresses commonly encountered in industrial and agricultural settings (Liu et al., 2023). The isolation of a novel strain from this niche is significant, as microbes from specialized environments often possess unique genetic and metabolic adaptations. This finding supports the broader principle of bioprospecting in industrial waste streams, which can yield microbial strains with enhanced or novel functionalities for biotechnology (Maina et al., 2017). Valorizing CSL in this manner contributes to a circular bioeconomy by transforming a low-value byproduct into a source of high-value microbial resources.

4.2. Taxonomic Clarification and Genomic Insights

The polyphasic approach employed in this study integrating classical phenotyping with modern molecular techniques provides a robust and unambiguous identification of strain IFO01 as Bacillus subtilis. The 16S rRNA gene sequence similarity of 99.10% firmly places it within this species, a threshold widely accepted for species delineation in bacteria (Chun & Rainey, 2014). The phylogenetic analysis offers more than just identification; it provides evolutionary context. The positioning of IFO01 on a distinct branch closely related to B. subtilis strain C3a-FIIRO, yet separate from other reference strains, suggests it represents a unique lineage within the species. This phylogenetic distinctness may correlate with phenotypic or metabolic differences acquired through adaptation to the CSL environment.

The discovery of an 887 bp plasmid in strain IFO01 adds an important dimension to its characterization. Plasmids in Bacillus species possess genes conferring special traits such as antibiotic resistance, heavy metal tolerance or the biosynthesis of secondary metabolites like antimicrobial peptides (Rückert et al., 2015). The encoded 288-amino-acid protein, with its distinctive composition rich in arginine and proline and potential for disulfide bond formation, suggests it may be a stable, secreted, or membrane-associated protein. Arginine-rich motifs are often found in DNA-binding domains or antimicrobial peptides, while proline can induce turns in protein structure. Although the precise function of this plasmid-borne gene requires experimental validation (e.g., through knockout studies), its presence indicates that strain IFO01 possesses accessory genetic elements that could contribute to its fitness and bioactivity. This finding underscores the importance of plasmid profiling in the complete genomic characterization of novel isolates, as these

mobile elements can be key drivers of functional diversity and biotechnological potential.

4.3. Bioactivity Potential and Preliminary Antimicrobial Assessment

The dose-dependent antimicrobial activity of the B. subtilis IFO01 whole-cell suspension is a promising indicator of its potential to produce bioactive compounds. The activity against an array of Gram-positive microorganisms suggests a broad-spectrum mechanism of action. This is characteristic of several B. subtilis-derived metabolites particularly the lipopeptide families (surfactin, iturin, fengycin), which are known to disrupt microbial membranes through detergent-like or pore-forming activities (Ongena & Jacques, 2008). The particularly strong inhibition of Streptococcus pneumoniae is noteworthy as respiratory infections caused by drug-resistant pneumococci remain a major global health challenge (Turner et al., 2019). The good activity against Proteus vulgaris, usually implicated in urinary tract infections and associated with biofilm formation and stone formation, is also of clinical relevance.

It is important to interpret these preliminary agar diffusion results with appropriate caution. The assay measures the combined effect of any extracellular metabolites present in the culture supernatant along with potential effects from whole cells. The activity observed is likely attributable to a cocktail of compounds rather than a single purified entity. Furthermore, the moderate activity against the pan-resistant clinical S. aureus isolate highlights a common challenge which is highly resistant pathogens with multiple, redundant resistance mechanisms (e.g., efflux pumps, enzyme production, target modification) are often less vulnerable to single antibacterial agents or crude extracts (Blair et al., 2015). This does not diminish the value of the finding but rather points to the need for subsequent steps: purification of the active compounds, determination of their minimum inhibitory concentrations (MICs) via broth microdilution, and investigation of potential synergy with conventional antibiotics.

4.4. Implications for Biotechnology and Future Directions

The isolation and characterization of B. subtilis IFO01 establishes a foundational resource with multiple potential biotechnological pathways. First, as a putative producer of antimicrobial substances, it serves as a starting point for drug discovery pipelines aimed at combating AMR. The logical next steps include optimization of fermentation conditions to maximize metabolite production, followed by bioactivity-guided fractionation to isolate and chemically characterize the active principles using techniques like HPLC, MS, and NMR. Second, beyond antimicrobials, B. subtilis strains are prolific producers of industrial enzymes (amylases, proteases, cellulases). The genetic adaptability suggested by its unique plasmid may be exploited through metabolic engineering to enhance the production of specific enzymes or other valuable compounds (Liu et al., 2023). Third, with its GRAS status, this strain or its metabolites could be explored for applications in food preservation, agriculture as a biocontrol agent, or even in probiotic formulations.

Future research should be directed along several key areas such as a step-by-step process to unravel specific mode of action of the antimicrobial constituents, potentially involving membrane integrity assays, genomic analysis for biosynthetic gene clusters, and transcriptomic responses in target pathogens. Scale-up and formulation studies will be crucial for any applied use. Safety assessment, including cytotoxicity and hemolysis assays, is essential to evaluate biocompatibility. Finally, exploring the synergistic potential of IFO01 metabolites with existing antibiotics, as suggested by the preliminary data showing complementary activity profiles, could lead to effective combination therapies that extend the lifespan of current drugs.

V. CONCLUSION

This study isolated and characterized a novel variant of Bacillus subtilis, designated IFO01, from corn steep liquor, an abundant agricultural byproduct. Through a rigorous polyphasic approach combining phenotypic analysis with 16S rRNA gene sequencing (GenBank OR052251) and plasmid profiling, the strain was conclusively identified and found to possess a unique genetic signature. Preliminary screening revealed that a suspension of this strain exhibits dose-dependent, broad-spectrum antimicrobial activity against several multidrug-resistant pathogens of clinical significance, with

notable efficacy against Streptococcus pneumoniae and Proteus vulgaris.

The work underscores the value of exploring underexplored ecological niches, such as industrial food-processing wastes, for discovering novel microbial resources with biotechnological potential. By valorizing corn steep liquor in this manner, the research contributes to the principles of a circular bioeconomy. Strain IFO01 represents a promising candidate for further investigation as a source of novel antimicrobial compounds, industrial enzymes, or other bioactive metabolites. Future work will focus on the purification and structural elucidation of the active principles, optimization of their production, and detailed evaluation of their efficacy and safety, paving the way for potential applications in addressing the critical challenge of antimicrobial resistance and in other industrial biotechnology sectors.

Authors Contribution:

1. Raji-Idowu, F.O. was involved in study design, data collection, statistical analysis, data interpretation, manuscript preparation, literature search and fund collection
2. Sanusi, J.F. was involved in study design, statistical analysis, data interpretation and manuscript preparation
3. Sirajudeen, A.O. was involved in study design, statistical analysis, data interpretation and manuscript preparation
4. Olanlege, A.O. was involved in statistical analysis, data interpretation, manuscript preparation, literature search

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Data Availability Statement: The 16S rRNA sequences generated in this study have been deposited in GenBank. All other data are available upon reasonable request from the corresponding author.

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