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Research Article

BIOHYDROGEN PRODUCTION THROUGH DARK FERMENTATION BY SOIL-DERIVED *BACILLUS PUMILUS*

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ABSTRACT

The search for renewable and eco-friendly energy alternatives has led to increased interest in microbial hydrogen production. In this study, soil samples from agricultural fields were analysed to isolate hydrogen-producing bacteria. The potential isolate, identified as *Bacillus pumilus*, exhibited strong amylase activity, enabling effective degradation of complex carbohydrates into fermentable sugars for dark fermentation. Hydrogen generation was carried out using organic waste substrates under controlled anaerobic conditions. GC–MS analysis confirmed hydrogen as the predominant gaseous product with minimal methane contamination. These findings demonstrate the capability of *Bacillus pumilus* as a robust, low-cost, and sustainable candidate for biological hydrogen production using agricultural residues.

Keywords: Biohydrogen, Dark fermentation, *Bacillus pumilus*, Amylase, Anaerobic bioreactor, Renewable energy.

INTRODUCTION

The global demand for sustainable energy has intensified the exploration of biohydrogen as a clean, renewable fuel. Among various biological routes, dark fermentation (DF) an anaerobic process converting organic substrates into hydrogen and volatile fatty acids offers high productivity, light independence, and compatibility with waste valorization (Yadav *et al*., 2023; Silva-Martínez *et al*., 2025). The process is primarily governed by hydrogenase and formate hydrogen-lyase (FHL) enzyme systems, which catalyze proton reduction during carbohydrate metabolism (Ghimire *et al*., 2015). In DF, hydrogen is produced as a metabolic by-product of carbohydrate degradation under anaerobic conditions. The mechanism is primarily mediated by hydrogenase and formate hydrogen-lyase (FHL) enzyme systems. In the ferredoxin-linked pathway, pyruvate-ferredoxin oxidoreductase (PFOR) oxidizes pyruvate to acetyl-CoA, transferring electrons to ferredoxin; reduced ferredoxin then donates electrons to [FeFe]-hydrogenase, releasing H_2 (Ghimire *et al*., 2015). Alternatively, in the PFL/FHL route, pyruvate formate-lyase converts pyruvate to formate, which is oxidized by FHL to produce H_2 and CO_2 (Patel *et al*., 2010). The balance between these enzymatic routes determines the yield and pattern of end products such as acetate, butyrate, ethanol, and lactate (Yadav *et al.,* 2023).

Although *Clostridium* species are classical hydrogen producers, recent research has highlighted facultative anaerobes such as *Bacillus* spp. for their stability, enzyme productivity, and environmental tolerance (Albuquerque et al., 2024). *Bacillus pumilus*, a spore-forming soil bacterium, is known for its hydrolytic enzyme secretion, including amylase, xylanase, and protease, supporting substrate degradation during fermentation (Menshawy *et al.,* 2025; Pan *et al*., 2023). Among these, *Bacillus pumilus* is an ecologically versatile soil and rhizosphere bacterium recognized for its metabolic diversity and enzyme productivity, including cellulases, xylanases, and proteases (Menshawy *et al.,* 2025; Pan *et al*., 2023a). Studies have reported that *B. pumilus* strains can adapt to a broad pH and temperature range, produce significant hydrolytic activity, and withstand oxidative stress (PLOS ONE, 2014). These

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physiological traits make *B. pumilus* a suitable candidate for DF processes where pre-treatment and hydrolysis of complex feedstocks are critical. Moreover, its facultative metabolism allows it to consume residual oxygen in the fermentation system, creating anaerobic microenvironments favorable for H_2 evolution (Pan *et al*., 2023b).

Emerging genomic analyses indicate that *B. pumilus* harbors genes encoding [FeFe]-hydrogenase-like proteins and related redox enzymes, suggesting potential involvement in fermentative hydrogen metabolism. Although the hydrogen yields of *B. pumilus* are typically lower than those of strict anaerobes, strain-specific variations and synergistic effects in co-cultures can substantially improve production (Patel *et al.,* 2010; Silva-Martínez *et al*., 2025). Additionally, *B. pumilus* can enhance system performance by producing hydrolytic enzymes that liberate fermentable sugars from complex biomass, thereby facilitating secondary hydrogen production by other microbes (Yadav *et al*., 2023). Hence, this study aimed to isolate and characterize soil-derived *Bacillus pumilus* and evaluate its biohydrogen production through dark fermentation using organic waste substrates. The work focuses on the microbial, enzymatic, and analytical aspects of hydrogen generation, contributing to sustainable bioenergy research.

MATERIALS AND METHODS

Sample Collection and Isolation

Soil samples were collected aseptically from agricultural land and serially diluted (10^{-3}–10^{-5}). Aliquots were plated on nutrient agar and incubated at 37 °C for 24 h. Distinct colonies were purified through repeated streaking (Pan *et al.,* 2023).

Morphological and Biochemical Characterization

The isolates were characterized by Gram staining, endospore staining, and motility tests. Standard biochemical assays indole, MR-VP, citrate utilization, catalase, oxidase, and carbohydrate fermentation were conducted as per standard methods (Cappuccino & Sherman, 2022; Yadav *et al*., 2023).

Screening for Amylase Activity

Amylase activity was tested on starch-casein agar by incubating isolates at 37 °C for 24 h and flooding with iodine solution. Clear zones indicated starch hydrolysis (Menshawy *et al*., 2025).

Molecular Identification

Genomic DNA was extracted using the Bionteq Gel Elution Kit. The 16S rRNA gene (~1.5 kb) was amplified using primers 27F/1492R (Ghimire et al., 2015). PCR products were sequenced and analyzed using Chromas Pro and Clustal X v2.0; sequences were compared with NCBI BLASTn for taxonomic identification. Phylogenetic trees were constructed using MEGA X (Silva-Martínez *et al*., 2025).

Substrate Preparation

Organic substrates such as fruit peels and agricultural residues were dried, powdered, and sieved (<1 mm). The biomass slurry (5% w/v) was autoclaved (121 °C, 15 min) to enhance hydrolysis and sterility (Albuquerque *et al*., 2024).

Dark Fermentation Setup

Batch experiments were carried out in 500 mL anaerobic reactors containing 300 mL fermentation medium (KH_2PO_4 1.5 g/L, K_2HPO_4 1.5 g/L, NH_4Cl 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, pH 6.0). The medium was flushed with nitrogen gas before inoculation (10% v/v *B. pumilus* culture). Fermentation proceeded at 37 °C and 150 rpm for 72 h (Yadav *et al*., 2023).

Gas Collection and Analysis

Evolved gases were collected in airtight balloons and analyzed using GC–MS (Shimadzu GC-2010 Plus) with an EC-5 column. Helium was used as carrier gas (2 mL/min) and oven temperature programmed from 35 °C to 450 °C. Hydrogen was identified by comparing retention times with standards (Ghimire *et al*., 2015).

RESULTS AND DISCUSSION

Serial dilution and nutrient agar plating of soil samples yielded several distinct bacterial colonies (Figure 1). Morphological examination showed the presence of both Gram-positive and Gram-negative bacteria, among which several isolates exhibited endospore formation and motility, typical features of the genus *Bacillus* (Pan *et al*., 2023). Biochemical profiling revealed marked diversity in metabolic activity, including differences in indole, MR-VP, citrate utilization, catalase, and oxidase tests (Table 1). Such diversity reflects the metabolic adaptability of soil microorganisms, enabling their survival in nutrient-variable environments (Cappuccino & Sherman, 2022). Among the isolates, a predominant strain exhibited strong amylolytic activity, forming clear hydrolytic zones on starch-casein agar plates after iodine flooding (Figure 2). The observed enzymatic activity is crucial for hydrolysis of polysaccharides, converting complex organic matter into fermentable sugars that serve as precursors for dark fermentation (Menshawy *et al*., 2025). The correlation between extracellular enzyme production and hydrogen yield has been well documented, as hydrolytic capacity directly enhances substrate availability and energy recovery (Yadav *et al*., 2023).

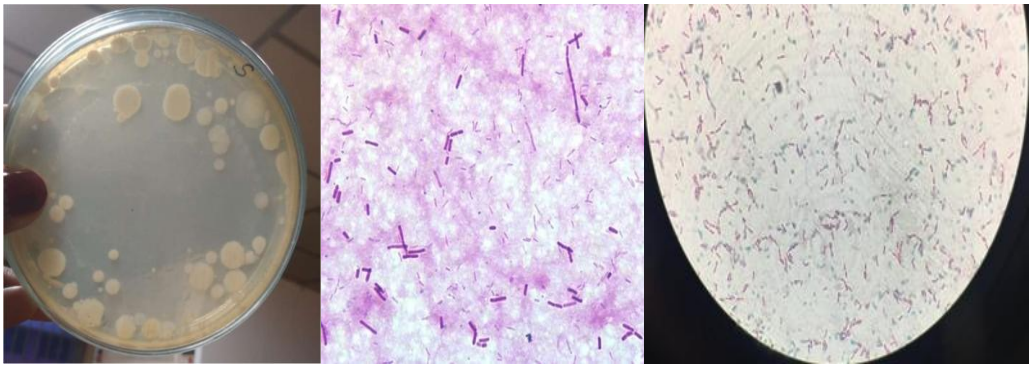


Figure 1. Isolation of bacterial colonies on nutrient agar and their microscopic observation.

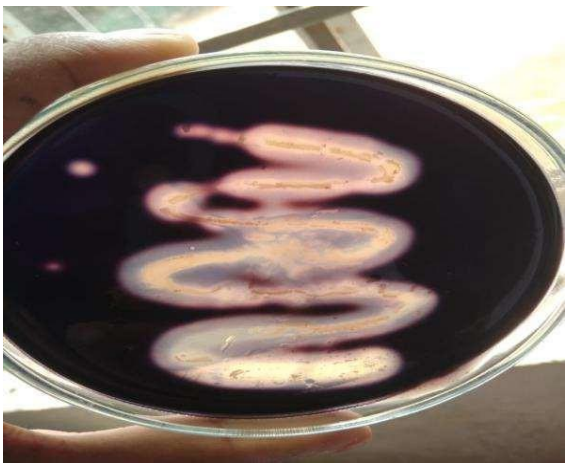


Figure 2. Amylase activity showing starch hydrolysis with zone.

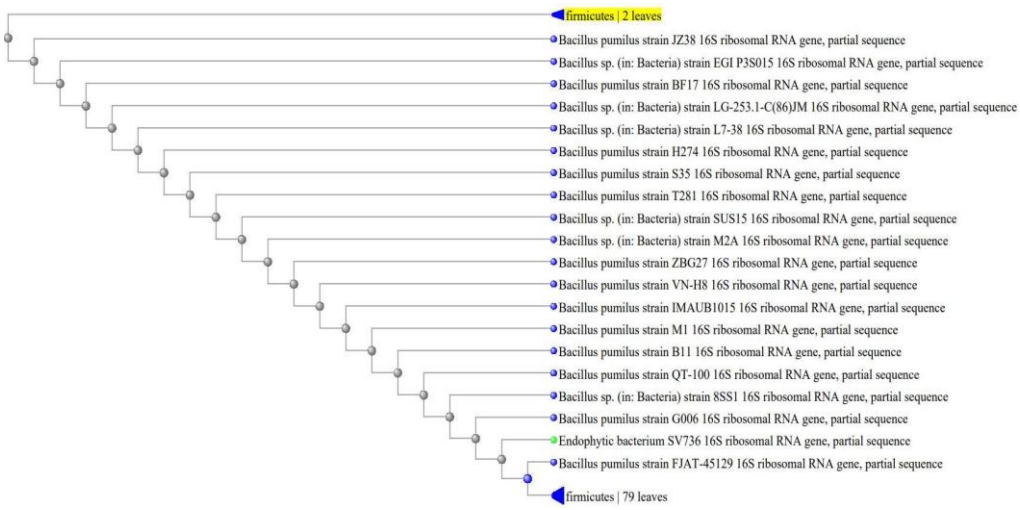
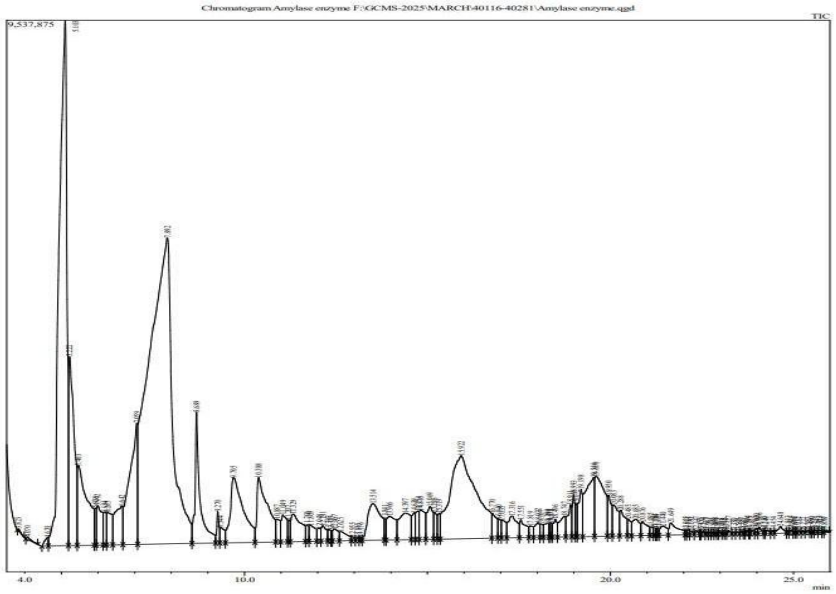


Figure 3. Phylogenetic tree of *Bacillus pumilus* isolate.



Peak Report TIC

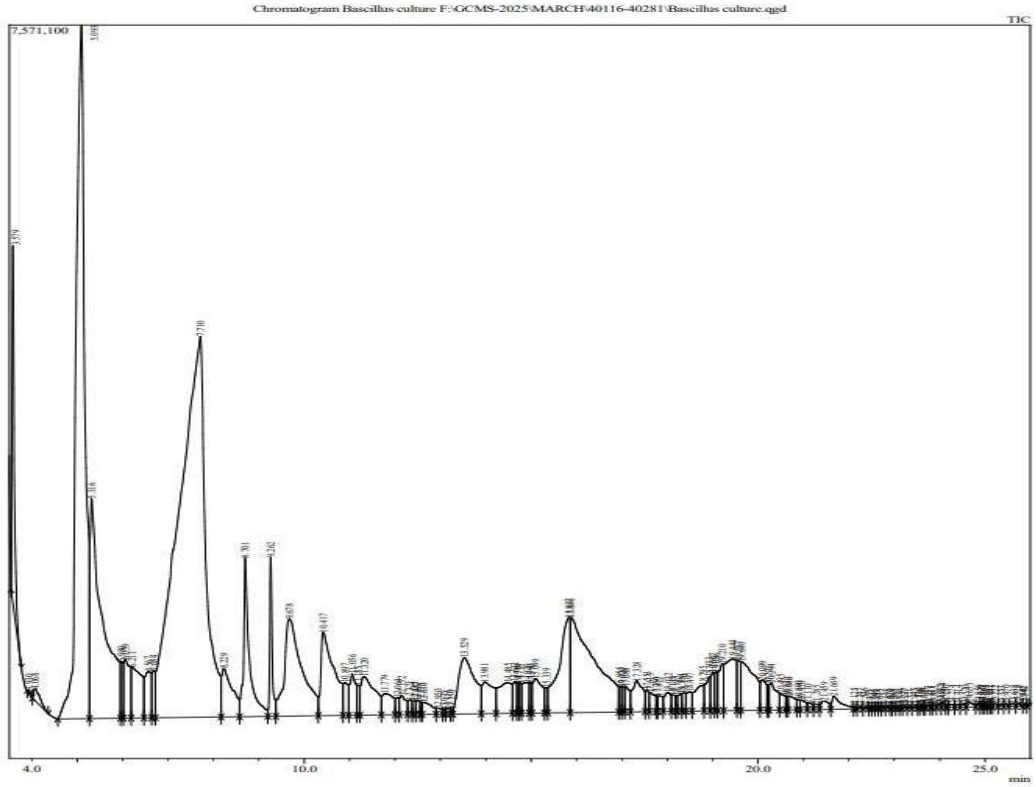
Peak#	R.Time	Area	Area%	Height	Name
1	3.823	177671	0.02	38899	6-Methyltetrahydro-1,3-oxazine-2-thione
2	4.070	355676	0.04	43762	BUTANOIC ACID, 3-METHYL-
3	4.620	969048	0.11	147016	2-Furanmethanol
4	5.103	127908406	15.09	9084999	2-HYDROXYPROPANOIC ACID
5	5.222	33468518	3.95	3269974	2-Cyclopenten-1-one, 2-hydroxy-
6	5.463	25762304	3.04	1379708	2(3H)-FURANONE, 5-METHYL-
7	5.928	2096782	0.25	641160	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
8	5.992	6968350	0.82	681181	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
9	6.184	3024983	0.36	565315	PENTANOIC ACID, 2-METHYL-
10	6.265	5693406	0.67	559719	1,2,4-TRIAZOLE, 4-[N-(2-HYDROXYETHYL)-N-NITRO]AMINO-
11	6.642	9976830	1.18	666209	Triethylenediamine
12	7.059	31312189	3.69	2097668	Pentanoic acid, 4-oxo-
13	7.892	234122625	27.63	5292582	1,2,3-PROPANETRIOL
14	8.689	21178603	2.50	2266665	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
15	9.270	2292090	0.27	543545	.alpha.-Terpineol
16	9.344	2264222	0.27	267081	Ethyl hydrogen succinate
17	9.705	28106976	3.32	1123990	Acetamide, N-(3-oxo-4-isoxazolidinyl)-, (R)-
18	10.388	22807383	2.69	1118502	1,2,3-Propanetriol, 1-acetate
19	10.887	3003182	0.35	400009	2,4-Dihydroxyacetophenone, 2TMS derivative
20	11.049	4860748	0.57	469012	CYCLOHEXANEMETHANOL, 4-HYDROXY-.ALPHA.,.ALPHA.,4-TRIME
21	11.207	1565967	0.18	381572	ETHANOL, 1,1'-OXYBIS-, DIACETATE
22	11.329	9223036	1.09	478157	2-propenoic acid, 2-[(1,3-dioxobutyl)amino]ethyl ester
23	11.706	1476282	0.17	278144	1,1-DIETHOXY-ETHANE
24	11.809	3204293	0.38	277862	5-O-Methyl-d-gluconic acid dimethylamide
25	12.048	1451830	0.17	223065	1-BUTANAMINE, 4,4-DIETHOXY-
26	12.141	2591430	0.31	270132	3-Octenoic acid, 4-methyl-2-pentyl ester
27	12.307	971742	0.11	195131	1,3:2,5-Dimethylene-l-rhamnitol
28	12.375	442291	0.05	184498	3-Mercapto-3-methyl-1-hexanol
29	12.467	2167886	0.26	208266	1-OCTENE
30	12.625	2280334	0.27	161311	6,8-DIOXABICYCLO(3.2.1)OCTAN-4.BETA.-OL
31	12.955	398305	0.05	67470	Acetonylacetone dioxime
32	13.105	330658	0.04	53880	PENTANOIC ACID, 2-METHYL-4-OXO-, ETHYL ESTER
33	13.176	273430	0.03	60579	TRIMETHYLSILYL ESTER OF TETRACOSANOIC ACID
34	13.514	15469770	1.83	634357	2-AMINO-9-(3,4-DIHYDROXY-5-HYDROXYMETHYL-TETRAHYDRO-F
35	13.841	1095892	0.13	367855	Nonanoic acid, hexyl ester
36	13.966	6716137	0.79	411141	Methyl 6-O-[1-methylpropyl]-.beta.-d-galactopyranoside
37	14.397	9930355	1.17	461898	3-Methyl-1-diisopropylsilyloxybutane
38	14.639	2787926	0.33	464830	9-Oxabicyclo[3.3.1]nonane-2,6-diol
39	14.754	3176447	0.37	492934	Diethylene glycol monododecyl ether
40	14.816	5229162	0.62	503217	d-Glycero-d-galacto-heptose
41	15.069	6510021	0.77	564619	1,2,3-PROPANETRIOL, TRIACETATE
42	15.196	2667082	0.31	467950	1,3,4-thiadiazole-2,5-diamine, N2,N2,N5,N5-tetramethyl-
43	15.319	2083676	0.25	447498	Pentetic Acid
44	15.922	72948471	8.61	1441142	3-Deoxy-d-mannoic lactone
45	16.770	3722788	0.44	430243	1,2-Di(prop-2-enyl)-tetramethyldisilane
46	16.940	1734102	0.20	334101	2-Ethylhexyl trimethylsilyl methylphosphonate
47	17.035	2628189	0.31	308263	(1S,14S)-Bicyclo[12.10.0]-3,6,9,12,15,18,21,24-octaoxatetracosane
48	17.316	6350843	0.75	378321	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
49	17.551	3621132	0.43	316273	Neophytadiene
50	17.819	1507776	0.18	202769	3,7,11,15-Tetramethyl-2-hexadecen-1-ol

Figure 4. GC-MS chromatogram of hydrogen gas: Showing the GCMS peak for Biohydrogen production without enzyme.

Table 1. Biochemical characteristics of isolates.

Biochemical Test	Indole	MR Test	VP Test	Citrate Test	Oxidase	Catalse	Urease Test
Colony 1	+	-	+	-	+	+	-

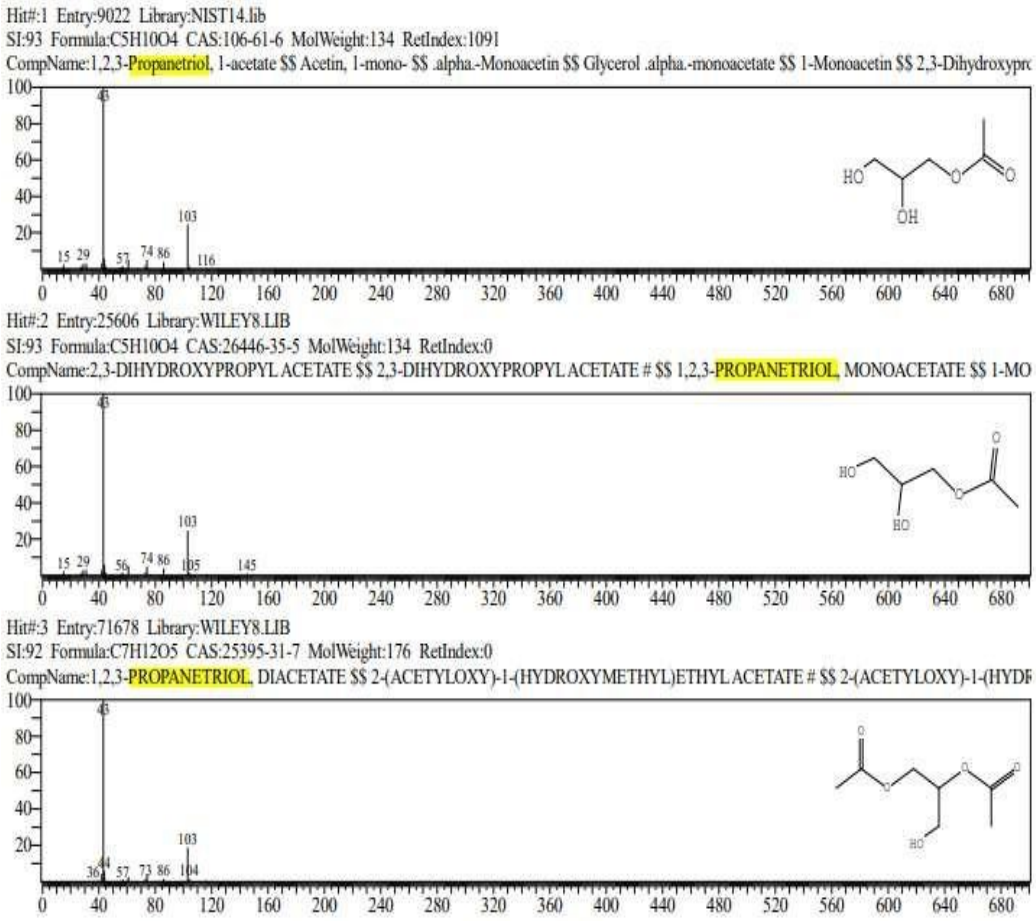
(+) Symbols indicate positive and (-) symbol indicate negative



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Name
1	3.579	12125509	2.00	3689844	2,3-Butanediol, [R-(R*,R*)]-
2	3.930	93825	0.02	29361	4-{2-[(4-AMINO-1,2,5-OXADIAZOL-3-YL)OXY]ETHOXY}-1,2,5-OXADI
3	4.068	1230733	0.20	120504	Butanoic acid, 3-methyl-
4	5.093	94692693	15.66	7159497	2-HYDROXYPROPANOIC ACID
5	5.316	43956053	7.27	2272797	2-Cyclopenten-1-one, 2-hydroxy-
6	5.980	2426191	0.40	586169	1,3-Dioxolane, 2,4,5-trimethyl-
7	6.059	5704247	0.94	606249	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
8	6.211	7956777	1.32	525100	dl-Homoserine
9	6.567	4673482	0.77	475864	1-PENTENE, 2,4,4-TRIMETHYL-
10	6.684	2287928	0.38	483970	4(H)-Pyridine, N-acetyl-
11	7.710	160273634	26.50	3931460	1,2,3-PROPANETRIOL
12	8.229	8122535	1.34	497331	BENZENEETHANOL
13	8.701	14513542	2.40	1638511	2,3-DIHYDRO-3,5-DIHYDROXY-6-METHYL-4H-PYRAN-4-ONE
14	9.262	6031563	1.00	1641038	(+)-ALPHA-TERPINEOL (P-MENTH-1-EN-8-OL)
15	9.678	27111199	4.48	999353	Isosorbide Dinitrate
16	10.417	17093257	2.83	859457	1,2,3-Propanetriol, 1-acetate
17	10.897	2459852	0.41	340512	D-glycero-D-manno-Heptitol
18	11.056	3858763	0.64	430012	CYCLOHEXANEMETHANOL, 4-HYDROXY-.ALPHA.,.ALPHA.,4-TRIME
19	11.185	1287624	0.21	310340	(2RS,3SR,4RS)-2,3 : 4,5-DIEPOXY-2,5-DIMETHYLHEXANAMIDE
20	11.320	8445371	1.40	397979	1,3-DIOXOLANE, 2-ETHENYL-2,4-DIMETHYL-, TRANS-
21	11.779	3662229	0.61	219608	1,3-DIETHOXYBUTANE
22	12.064	939627	0.16	176883	Formic acid, 2-(2-methoxyethyl)hexyl ester
23	12.152	1862308	0.31	199330	HEXADECANE
24	12.321	873266	0.14	152255	METHYL 2,3,6,7-TETRA-O-ACETYL-4-O-METHYLHEPTOPYRANOSID
25	12.435	628353	0.10	140811	D-RIBOFURANOSE, 2,3-O-(1-METHYLETHYLIDENE)-
26	12.487	830578	0.14	144353	2-Acetoxytetradecane
27	12.575	344112	0.06	128131	1,3,5-TRIAZINE-2,4(1H,3H)-DIONE, 6-(METHYLAMINO)-
28	12.630	1781453	0.29	125401	1-Butanol, 4-(butylnitrosoamino)-
29	12.950	493707	0.08	62177	1R-Ethoxy-3-cis-methoxy-2-cis-methylcyclohexane
30	13.075	182695	0.03	47276	1(4H)-NAPHTHALENONE, 4A,5,8,8A-TETRAHYDRO-4-HYDROXY-, (4.
31	13.176	323989	0.05	54861	Hexanohydrazide, 2-butyl-2-hydroxy-N2-(3-phenylpropenylideno)-
32	13.250	162826	0.03	49772	N-[3-(4-CHLORO-PHENYL)-5-ETHYLCARBAMOYLMETHYL-4-OXO-2-
33	13.529	14495940	2.40	580621	2-AMINO-9-(3,4-DIHYDROXY-5-HYDROXYMETHYL-TETRAHYDRO-F
34	13.981	5719913	0.95	326634	.BETA.-D-GLUCOPYRANOSE, 1,6-ANHYDRO-
35	14.485	6388010	1.06	320216	2,5-Monoformal-l-rhamnitol
36	14.641	1795069	0.30	326953	08217205002 FLAVONOL 3',4',5,7-OH,3-O-ARAGLUCOSIDE
37	14.710	840316	0.14	313141	6-Nonenal, 3,7-dimethyl-
38	14.755	1110428	0.18	314145	DECANE
39	14.920	3168667	0.52	314253	4-Methylmannitol
40	14.990	1117458	0.18	312175	2R,3S-9-[1,3,4-Trihydroxy-2-butoxymethyl]guanine
41	15.099	5103520	0.84	356896	1,2,3-PROPANETRIOL, TRIACETATE
42	15.339	1090501	0.18	262923	5-METHYL-2-DIETHYLAMINO-2-THIAZOLINE
43	15.837	16731014	2.77	982001	3-Deoxy-d-mannoic lactone
44	15.886	36267219	6.00	980507	3-Deoxy-d-mannoic lactone
45	16.955	942741	0.16	264159	Cis-2,3-dimethylthiane
46	17.020	1078914	0.18	257651	2(1H)-ISOQUINOLINECARBOXAMIDINE, 3,4-DIHYDRO-
47	17.088	1863171	0.31	260018	Dodecane, 2-methyl-
48	17.328	5063026	0.84	320047	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
49	17.551	1083880	0.18	227208	Neophytadiene
50	17.615	1661451	0.27	198884	2-OCTENE, 1-BROMO-1,1,3-TRIFLUORO-

Graph 2. Showing the GCMS peak for Amylase Biohydrogen production



Graph 3. Showing the 1,2,3-PROPANETRIOL (Glycerol) structure.

Table 2. Hydrogen yield and composition of produced gas.

Isolate	Hydrogen (%)	Carbon Dioxide (%)	Methane (%)	Other Gases (%)
A	92.5	5.8	1.2	0.5
B	89.8	7.1	2.5	0.6
C	85.3	10.2	3.5	1

Table 3. Accession number obtained from GenBank.

Isolates	Molecular Identification	NCBI Accession number
ISN 1	*Bacillus pumilus*	PQ882319

The promising isolate was subjected to molecular identification through 16S rRNA gene sequencing, yielding an amplicon of approximately 1.5 kb. Sequence alignment and BLAST analysis confirmed the isolate as *Bacillus pumilus*, showing 99.2% similarity with reference sequences in the NCBI GenBank database (Figure 3). Phylogenetic tree construction using the neighbor-joining method further clustered the isolate with known *B. pumilus* strains, consistent with its morphological and biochemical characteristics (Silva-Martínez *et al*., 2025). The molecular confirmation validated the potential of *B. pumilus* as a facultative anaerobic hydrogen producer, aligning with previous reports highlighting its role in biohydrogen generation and polysaccharide degradation (Albuquerque *et al*., 2024). Batch dark fermentation experiments demonstrated that *B. pumilus* effectively converted organic substrates (fruit residues and agricultural biomass) into hydrogen under anaerobic conditions. Hydrogen gas was collected via gas lines into balloons and quantified using GC–MS analysis, which confirmed hydrogen as the predominant gaseous product, with negligible methane or carbon dioxide contamination (Figure 4; Table 2). The absence of methanogenesis indicated effective anaerobic control, as *Bacillus* spp. generally suppress methanogens by rapid acid production during early fermentation stages (Ghimire *et al*., 2015).

The hydrogen yield was enhanced by the amylase-mediated breakdown of polysaccharides, increasing the pool of fermentable sugars for hydrogenogenic metabolism. This enzymatic hydrolysis likely promoted efficient electron flow toward hydrogenase-mediated reactions, improving the overall yield. The [FeFe]-hydrogenase system in *B. pumilus* is believed to facilitate proton reduction to molecular hydrogen, consistent with observations from other facultative anaerobes (Silva-Martínez *et al*., 2025). Moreover, the strain maintained stable hydrogen production within a temperature range of 30–37 °C and pH 5.5–6.5, demonstrating adaptability to moderate fermentation conditions. These findings support the suitability of *B. pumilus* as a cost-effective and eco-friendly candidate for biological hydrogen production using renewable waste feedstocks (Yadav *et al.*, 2023; Albuquerque *et al*., 2024).

Compared with traditional strict anaerobes such as *Clostridium* spp., *Bacillus pumilus* offers advantages including oxygen tolerance, rapid growth, and spore-forming ability, simplifying inoculum preparation and reactor maintenance (Pan *et al*., 2023). The strain's capability to secrete extracellular enzymes further enables the conversion of raw organic waste without extensive pretreatment, reducing process costs. These characteristics make *B. pumilus* particularly valuable for rural-scale or decentralized biohydrogen systems, where inexpensive substrates such as fruit peels or agricultural residues are abundantly available. The integration of enzyme-rich *Bacillus* species in co-culture systems could further enhance substrate degradation and overall hydrogen productivity (Menshawy *et al*., 2025). Overall, the results demonstrate that soil-derived *Bacillus pumilus* is a robust and versatile biohydrogen producer capable of sustainable dark fermentation, aligning with current research goals toward green energy transition and circular bioeconomy development.

CONCLUSION

The present study successfully demonstrated the potential of soil-derived *Bacillus pumilus* for biohydrogen production through dark fermentation using organic waste substrates. Morphological, biochemical, and molecular analyses confirmed the identity and metabolic versatility of the isolate. The strong amylolytic activity of *B. pumilus* enabled effective hydrolysis of complex polysaccharides, thereby enhancing substrate utilization and hydrogen yield. GC–MS analysis verified hydrogen as the dominant gaseous product, indicating efficient fermentative metabolism under controlled anaerobic conditions. The ability of *B. pumilus* to tolerate moderate environmental fluctuations, coupled with its rapid growth and enzyme secretion capability, makes it an economically viable and eco-friendly alternative to traditional strict anaerobes. These findings emphasize the suitability of *B. pumilus* for sustainable hydrogen generation from agricultural and food wastes, contributing to waste valorization and renewable energy goals.

ACKNOWLEDGMENT

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

FUNDING

This study received no specific funding from public, commercial, or not-for-profit funding agencies.

AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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