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Isolation and Characterization of Lignocellulolytic Bacteria from Municipal Solid Waste Landfill for Identification of Potential Hydrolytic Enzyme

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Abstract: The utilization of lignocellulose biomass as an alternative source of renewable energy production via green technology is becoming important, and is in line with sustainable development goal initiatives. Lignocellulolytic bacteria, such as *Bacillus* spp., can break down biomass by producing hydrolytic enzymes, which are crucial in the successful conversion of biomass or lignocellulosic material into renewable energy. This information gave rise to this study, where municipal solid waste sediments of a sanitary municipal solid waste landfill were sampled and screened, and lignocellulolytic bacteria were isolated and characterized. Samples were taken from four different locations at the Pulau Burung landfill site in Malaysia. Lignin and starch were used as sources of carbon to identify potential bacteria that exhibit multi-enzymatic activity. The growth rate and doubling time of bacterial isolates in lignin and starch were taken as the criteria for selection. Eleven bacterial isolates were screened for cellulase activity using iodine and Congo red dyes. The cellulase activity of these isolates ranged from 0.8 to 1.7 U/mL. We carried out 16S rRNA gene sequencing to identify the phyla of the selected bacterial isolates. Phylogenetic analysis was also conducted based on the 16S rRNA sequences of the bacterial isolates and related Bacillus species, and a tree was generated using the Neighbor-Joining method. In this study, *Bacillus proteolyticus*, *Bacillus Sanguinis*, *Bacillus spizizenii*, *Bacillus paramycoides*, *Bacillus paranthracis and Neobacillus fumarioli* were identified as promising bacteria capable of expressing lignocellulolytic enzymes and degrading the lignocellulosic biomass present in municipal solid waste.

Keywords: *Bacillus* spp; cellulase; lignocellulose biomass; lignocellulolytic bacteria; municipal solid waste

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1. Introduction

The continuous rise in the global population and fast industrialization and urbanization have shown direct impacts on the production of municipal solid waste [1]. Municipal solid waste (MSW) has become a serious concern in the developing and least-developed nations, where adequate waste collection facilities are not available [2]. Approximately two billion metric tons of urban solid waste are currently generated at the global level, and this is expected to double by the year 2050 [3]. The disposal methods of municipal solid waste are not well planned globally, and this waste has become a major environmental threat

during the past few years [4]. Landfills have been adopted as a major means of disposing of municipal solid waste in both industrialized and developing countries [5]. Municipal solid waste disposal into landfill sites has had deleterious impacts on aquatic ecosystems and the health of human-beings due to its offensive odor, leachate leakage and toxic gas emissions [6–9].

The high demand for energy, together with the fast depletion rate of fossil fuels, is gaining global attention [10]. The energy transition is an important step towards carbon neutrality, and it is beneficial for developing countries in terms of environmental protection and the economy [11]. There is a need to move from fossil fuels to renewable energy sources to achieve the energy transition to low carbon generation. The waste disposal and energy crisis problems can be mitigated by exploiting lignocellulosic biomass, which is readily available at zero cost as a renewable resource [12]. Lignocellulose is a widely available and unexploited source, as around two hundred billion tons of lignocellulose are generated annually all across the globe [13]. Lignocellulose biomass consists of agricultural and forestry residues, yard trimmings and energy crops, which are made up of cellulose, hemicellulose and lignin and form a predominant part of the green waste fraction of municipal solid waste (MSW). Approximately 50 and 12% cellulose and hemicellulose, respectively, are present in residential municipal solid waste in the form of dry weight [14]. Lignocellulosic materials have become a major feedstock for biofuels because the presence of multi-carbon components and their derivatives can be transformed into value-added materials for the syntheses of sugar, alcohol, lipids, etc. [15,16]. Various physical and chemical methods have been applied in lignocellulose bioconversion, but due to their high costs, use of toxic chemicals, and complicated and expensive procedures, their processes are economically infeasible, and thus, these strategies have not been successful. The biodegradation of lignocellulose biomass by microbial enzymes is a promising and sustainable approach, as microbes can simultaneously perform the role of pre-treatment and easily break down lignocellulosic components [17]. Different types of enzyme, such as ligninolytic, cellulolytic and hemicellulolytic enzymes, can be applied in lignocellulose biodegradation [18]. Poszytek et al. [19], in 2016, reported greater efficiency of microbial lignocellulolytic enzymes for bioconversion compared to commercial enzymes. The individual microbial strains or consortia secrete hydrolytic enzymes during their metabolism and degrade cellulose, hemicellulose and lignin into smaller fragments [20]. A number of previous studies revealed that an anaerobic environment, such as the bovine rumen, and the elephant and termite gut, acts as a potential source of lignocellulose-degrading enzymes [21,22]. Such environments harbor microbial communities that convert lignocellulosic biomass without pre-treatment and are currently applied in commercial processes. The composition of leachate and sediment at landfill sites depends on different factors, such as the age of the landfill, its waste composition, its temperature, etc. [23]. The structure of a microbial community is also affected by the age and composition of the landfill.

Bacteria are considered potential candidates for industrial applications due to their fast growth, the presence of abundant enzymes, their pressure resistance and their ease of genetic manipulation to achieve improved properties [24,25]. Bacteria are prolific producers of cellulase and are extracellularly secreted in huge amounts. Cellulase is considered the black box of lignocellulose degradation, and utilizes the homogeneous property of cellulose by hydrolyzing β-1, 4 glycosidic linkages [26]. Various types of cellulose may incite bacteria to generate different types of cellulase and make microbes specific to lignocellulosic materials [27]. *Bacillus* is a potential genus of bacteria which significantly generates cellulase enzymes [28,29]. *Bacillus coagulans*, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus cereus* reflected high cellulolytic activity for lignocellulosic substances [30]. Balla et al. [31], in 2022, reported high cellulolytic activity in bacterial communities across different ecosystem, and observed that bacterial species were able to produce enzymes that can hydrolyze cellulosic substrates present in both soluble and insoluble states. Cellulase is composed of endoglucanases, exoglucanases, and β-glucosidases, which act in a synergistic way to hydrolyze the lignocellulosic biomass [32]. The endo-β-1, 4-glucanase and exo-β-1,4

cellobiohydrolase degrade cellulose into cello-oligosaccharides, which are then hydrolyzed to glucose by β-glucosidase [33].

Landfill sites are heterogeneous in nature, comprise mainly lignocellulosic material and can be considered ideal sites for biomass conversion. However, landfill sites have not been extensively explored and studied [34]. Studies have confirmed that *Bacillus* spp. secrete enzymes for lignin and cellulose degradation, metabolize dioxane lignin and break the biphenyl structures of lignin. *Clostridium*, *Cellulomonas Rumminococcus*, *Alteromonas*, *Acetivibrio, etc.* are other bacteria that have been reported to exhibit cellulolytic activity. Due to their fast propagation, convenient molecular genetics, protein expression with a smaller genome and high adaptability towards harsh environmental conditions, bacteria are suitable candidates for the degradation of lignocellulosic biomass [26]. Yong et al. [35], in 2019, reviewed the status of municipal solid waste in Malaysia and reported that the application of MSW in energy generation may promote growth and sustainable development in Malaysia. To the best of our knowledge, no reports are available in the literature regarding the isolation and characterization of potential bacteria and their application in lignocellulose biomass conversion at the Pulau Burung landfill site in Malaysia. Therefore, we were interested in finding potential candidates that can efficiently degrade the cellulose and hemicellulose contents of the lignocellulosic materials present in MSW. The main objectives of the present investigation were: (i) the extensive characterization and identification of lignocellulolytic bacteria present at the Pulau Burung landfill site in Malaysia and their comparison with the existing literature data; (ii) the screening of multi-enzymatic bacteria that will make them more suitable in lignocellulose-driven refinery; and (iii) the generation of information and recommendations for designing future consortia for the complete degradation of lignocellulose.

2. Materials and Methods

2.1. Sampling and Physico-Chemical Characterization of Waste Samples

Sediment samples were collected from the Pulau Burung sanitary landfill site (5°19.36; 100°42.67′ E) located in Nibong Tebal, Penang, Malaysia, on 7 March 2020 at 9:45 a.m. The collected samples were transported to our laboratory as per the standard procedures described by Forster [36] for physico-chemical and microbiological analysis.

Sampling was performed during the rainy season in March 2020 from four different randomly selected sites at the Pulau Burung sanitary landfill, namely A, B, C and D, at a depth of 20 cm. The temperature was measured in situ at each site using a digital thermometer (Rapitest, Kuala Lumpur, Malaysia). Samples were kept at 4 °C until analysis.

pH was measured in the laboratory using the standard method described by Radojevic and Bashki [37]. Twenty grams of sediment that was free from larger materials was placed in a beaker. Forty milliliters of distilled water was mixed, and contents swirled and allowed to stand for 30 min. The pH was analyzed by using a pH meter (Mettler-Toledo, Zurich, Switzerland) by ensuring that the electrode did not touch the settled sediment particles but remained in the supernatant liquid above while the reading was taken. This prevented errors in the readings [37].

2.2. Isolation and Identification of Bacteria

2.2.1. Isolation of Potential Bacteria

Bacterial species were isolated as per the method of Reynolds [38]. The samples were serially diluted by weighing 1 g of sediment and diluting it tenfold. Afterwards, 0.1 mL of dilutions from each fold were dispersed on the sterilized nutrient agar plates and incubated at 37 °C for 24 h. The clear colonies were sub-cultured several times to ensure purity. The various colonies observed were sub-cultured using the streak plate method until pure colonies were obtained. The morphologies of the colonies were observed; those with similar morphologies were considered the same, and distinct ones were further sub-cultured to obtain pure colonies. Pure colonies were stored at 4 °C on nutrient agar slants for further analysis.

2.2.2. *Preliminary Identification of Potential Bacteria*

After the isolation of bacteria, morphological and biochemical characterization was conducted using standard procedures described in *Bergey's Manual of Determinative Bacteriology* for the identification of potential bacteria [39]. Morphological characterization was performed based on visual appearance and Gram staining using the method described by Smith [40].

2.3. Screening for Ligninolytic and Cellulolytic Ability

Predictive modeling was used to check the growth rate of microorganisms. This was measured by taking aliquots at intervals while growing the microbial culture [41]. The bacterial isolates were inoculated into pre-prepared sterilized media containing starch and lignin as the sole sources of nutrients. The composition of the media used is stated in Table 1. They were prepared to determine the lignocellulolytic ability of the bacterial isolates.

Table 1. Screening media composition.

	Deionized Water (mL)	**Sole Carbon Source (g)**
Lignin	10	0.1
Starch	10	0.1

The absorbance was taken at 600 nm using a Hach DR 2800 spectrophotometer (Hach Malaysia, Kuala Lumpur)hourly for up to 24 h. The results were used for kinetic growth studies of the bacterial population.

2.4. Kinetic Growth Studies for Ligninolytic and Cellulolytic Ability of Isolated Bacteria

Using the results from the screening (2.3), growth curves were derived and used to calculate growth rate and doubling time for each isolate. Mathematical modeling was used to fit the results obtained for bacterial growth curve prediction [42]. A positive fit indicated bacteria growth (G), while a negative fit was an indication of non-bacterial growth (NG). The coefficient of determination (R^2) was applied to analyze the efficiency of model. An R^2 value close to 1 indicated that the method is reliable for predicting the growth profile of the isolated bacteria.

To obtain the growth rate of the isolates, individual growth curves were processed by retrieving sequential sets of n data values, where n was in the range of 3 to 10, as previously described by Breidt et al. [43] in 1994. The slope of the line (I) was used to derive the growth rate, and the maximum slope is the specific growth rate. Values with higher R^2 were preferred, as they were more indicative of growth rate. Equations (1) and (2) as seen below depict the formula used for the kinetic studies:

$$Y = \mu \times Yo \quad (1)$$

μ = Growth rate

The formula for population growth rate, and duplication time is shown below:

$$TD = \frac{\ln 2}{K} \quad (2)$$

Doubling time (TD)

ln2 = Neperian logarithm of 2

k = Growth rate

2.5. Enzyme Assays

2.5.1. Qualitative Screening for Hydrolytic Enzyme Production

Upon completion of the kinetic studies, isolates that showed great potential in utilizing both lignin and starch were subjected to qualitative assays to test for xylanase, protease, amylase and cellulase. This potential was measured by comparing the growth rate (μ) in each medium with isolates that had better growth rates. For the specific isolation of cellulolytic microorganisms, CMC agar plates were prepared using the following composition: agar powder: 15 g, yeast extract: 1 g, CMC: 3 g, $FeSO_4.7H_2O$: 0.01 g, $(NH4)_2SO_4$: 1 g, NaCl: 2 g, $MgSO_4.7H_2O$: 0.2 g and KH_2PO_4: 1.36 g [44]. The plates were incubated at 37 °C for 3–5 days. Once single colonies had been observed, subsequent plates were split into quadrants. Colonies were placed in the middle of each quadrant and incubated once more.

For hydrolysis testing, the plates were flooded with freshly prepared Gram's iodine (2 g potassium iodine in 1 g of iodine dissolved in distilled water). The plates were read immediately, as the clear zone around the bacterial colony showed that hydrolysis was taking place. Positive plates gradually hydrolyzed the iodine until the plate became clear, whereas negative plates were unable to produce a clear zone around the bacterial colony, which reflected that there was no hydrolysis [44].

For the estimation of xylanase activity, colonies in the quadrants were flooded with Congo red (0.1% *w/v*), and then, de-stained with sodium chloride (0.1 M). The colonies that had clear zones around them were positive for xylanase, while those without it were considered negative.

To test for proteolytic ability, skimmed milk agar was prepared using the following composition: skimmed milk powder: 2.8 g, casinenzymic hydrolysates: 500 mg, yeast extract: 250 mg, dextrose: 100 mg and agar: 1.5 g (added to distilled water (100 mL) to make 1% skim milk agar). The agar plates were divided into quadrants, and isolates of interest were incubated and observed for 72 h, as per the method of Masi et al. [45]. Microbes that were able to grow showed proteolytic ability and formed halos around the colonies.

2.5.2. Quantitative Screening for Cellulolytic Ability of Microorganisms

The enzyme activity of cellulase for the DH13, DG6, AB7 and A3 strains was assayed by reducing the sugar content by Dinitrosalicylic acid [46]. These isolates were chosen because they showed enzyme activity for all qualitatively measured enzymes. Absorbance was measured at 540 nm and an enzyme unit (U) was expressed as the enzyme amount that released 1 μmol of glucose equivalent from carboxymethylcellulose.

2.6. DNA Extraction and Molecular Characterization

The DNA from pure isolates was extracted using a Vivantis DNA kit (Vivantis, Selangor, Malaysia), as per the standard method of Yi et al. [47]. The isolates were grown overnight in nutrient broth and kept in a shaker at 150 rpm and 37 °C. The obtained DNA was amplified through a polymerase chain reaction (PCR) using the universal DNA forward primer Eubac27F (50-AGAGTTTGATCCTGGCTC AG-30) and reverse primer 1492R (GGTTACCTTGTTAC GACTT-30) to target bacterial 16S rRNA. The PCR had the following protocol: 3 min at 95 °C for 32 cycles, 1 min at 94 °C, 1 min at 56 °C, 2 min for 72 °C, and 10 min for 72 °C with 4 °C intervals. After sequencing, the obtained sequences were blasted using the online tool NCBI Blast.

Phylogenetic Analysis

After blasting, the GenBank database was used to compare the 16S rDNA sequences with similar sequences. The phylogeny of the bacterial strains was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software.

2.7. Statistical Analysis

All the experiments were carried out in triplicate and data obtained in the form of mean ± standard error. Data were subjected to a two-factor analysis of variance (ANOVA) test using Microsoft excel, with significance levels of $p < 0.05$.

3. Results and Discussion

In the present investigation, sediment samples were collected from the Pulau Burung sanitary landfill in Penang, Malaysia, as shown in Table 2.

Table 2. Sampling location and condition of sediment samples collected from the Pulau Burung landfill.

Sampling Point	Latitude	Longitude	pH	Physical Appearance of Sediment	Temperature, °C
A	N5°12′6.9″	E100°25′24.7″	5.97	black	32
B	N5°12′14.8″	E100°25′33.8″	6.32	red	35
C	N5°12′7.6″	E100°25′26.3″	6.3	brown	36
D	N5°11′57.7″	E100°25′36.0″	6.9	loamy	28

pH gives an indication of the acidity or alkalinity of an environment and helps in understanding environmental interactions. The pH ranged from 5.97 to 6.9; site A showed the lowest and site D reflected highest pH, as seen in Table 1. The average pH was 6.37, which was close to the pH reported in earlier landfill studies conducted in Malaysia. pH is an important factor as it affects the possible outcome of any pretreatment process used. It has been observed that lower pH favors the hydrolysis of hemicellulose, and higher pH enhances the hydrolysis of lignin components [48].

The temperature was in the range of 28–36 °C; the site D had lowest, whereas site C had highest temperature. The average temperature was 32.8 °C. Temperature affects reactions in the environment and can amplify odor at a landfill site [49]. The variation in physio-chemical properties is due to the heterogeneity of the waste materials that are disposed of in the landfill. Saha et al. [50] reported that with increasing temperature, various gases, such as ammonia and methane, also generated.

3.1. Identification and Isolation of Bacteria

In total, 169 isolates were obtained based on the morphological characterization. A total of 37 cultures were isolated from sample site A, 34 from sample site B and 43 from sample site C, and a maximum of 55 were isolated from sample site D.

The isolates were visually and microscopically observed for characterization purposes. The Gram staining results and details of the colony features of the bacteria are highlighted in Table 3, and Figure 1 shows the appearance of some isolates after Gram staining.

These results reflect that 30% of the isolates were Gram-negative with varying shapes and arrangements (Figure 1b,c), whereas the other 70% were Gram-positive, as seen in Figure 1a,d. Our results are consistent with the findings of Zhai et al. [51] where the proportion of Gram-positive bacteria was greater compared to that of Gram-negative bacteria.

3.2. Screening for Lignocellulolytic Ability and Kinetic Studies

The isolates were tested to evaluate their ligninolytic and cellulolytic properties. The isolates were introduced into media containing either lignin or starch to analyze which isolate could grow with only one of these as a carbon source. The results reflected that out of the 169 isolates, only one isolate was unable to grow in either lignin or starch. Only 44 isolates could grow in either of the nutrient sources, so the kinetic studies focused on the 124 isolates that could grow in both lignin and starch (Supplementary Materials Table S1).

With the screening results, the growth curves, as seen in Figure 2, were derived to calculate the growth rate and doubling time for each isolate. The study of microbial growth

curves is an integral part of predictive microbiology and is used in various fields, as it allows for the integration of statistical, mathematical and microbiological principles in quantifying a microorganism's behavior [52]. In the modeling of bacterial growth kinetics, the behavior of a microorganism can be described under specific environmental conditions [42]. In this case, the bacterial isolates were grown in the same media of starch and lignin as sole sources of carbon, and incubated under the same conditions at 37 °C.

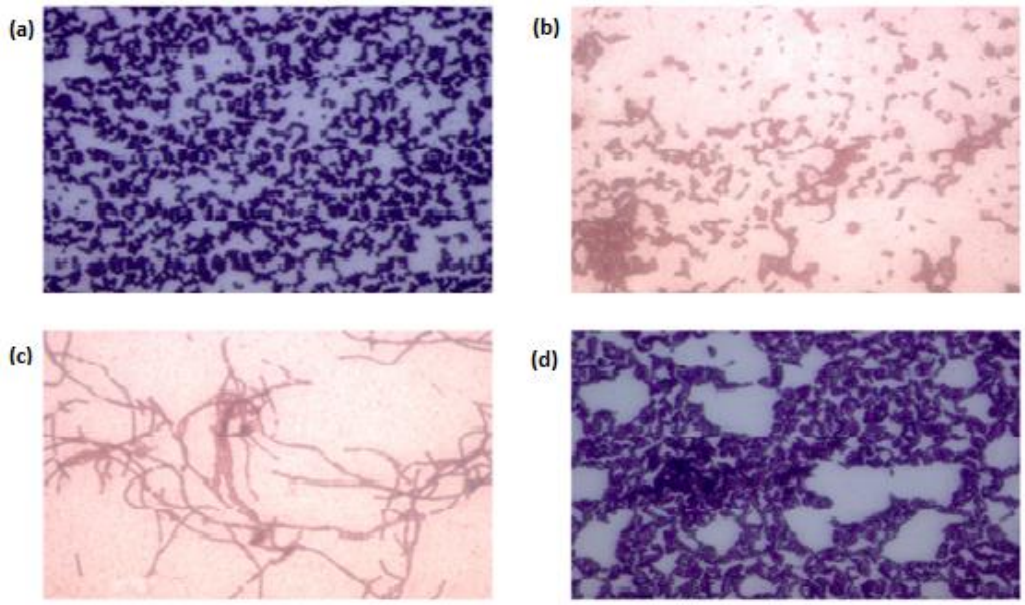

Figure 1. Gram staining of isolates for morphological identification. (**a**,**d**) Purple-colored Gram-positive bacterial isolates; (**b**,**c**) pink-colored Gram-negative bacterial isolates.

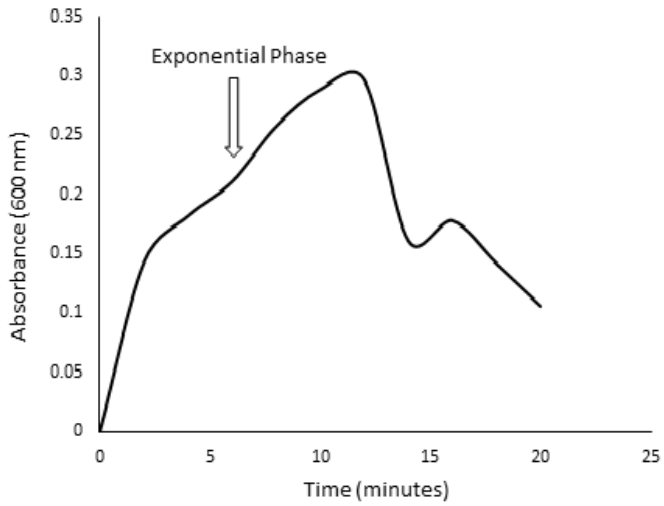

Figure 2. Growth Curve of a typical bacterial isolate from the experiment, showing the exponential phase, which was used to derive the data sets and growth rates.

The results obtained were fitted; a positive fit was likely an indication of bacterial growth, whereas a negative fit was an indication of non-bacterial growth. From the correlation equation, the growth rate was derived, and then, the doubling time (TD), which refers to the time it takes for the bacterial population to double during the exponential phase, was also derived. Only the isolates that showed a positive growth rate were taken into consideration, and their doubling time was also determined. The slope of the line (I) was used in determining the isolates' growth rate, and maximum slope connotes the

specific growth rate, which is depicted in Figure 3. The results are summarized in Table 3. Dey et al. [53] reported that there is a decrease in the duration time as the growth rate increases.

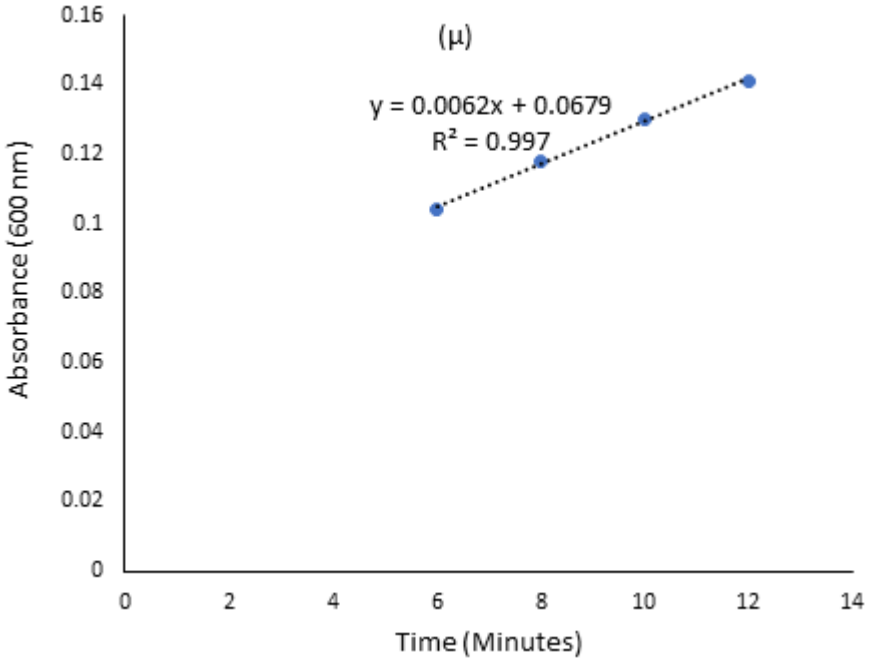

Figure 3. Slope of the growth curve showing the maximum growth rate of a typical bacterial isolate from the experiment.

Table 3. Results of kinetic studies showing growth rate and doubling time.

Starch				Lignin			
Strain	**Growth Rate (μ)**	**Doubling Time (min)**	**R^2**	**Strain**	**Growth Rate (μ)**	**Doubling Time (min)**	**R^2**
A1	0.0041	169.0603	0.8229	A1	0.0048	144.4057	0.7293
A17	0.0039	177.73	0.6297	A17	0.0121	57.28489	0.6275
A19	0.0415	16.70234	0.6025	A19	0.0184	37.67104	0.8382
A2	0.0027	256.7212	0.7946	A2	0.0024	288.8113	0.6095
A4	0.0092	75.34208	0.9605	A4	0.0075	92.41962	0.6279
A6	0.0075	92.41962	0.6614	A6	0.0095	72.96286	0.8324
A8	0.008	86.6434	0.9046	A8	0.0099	70.01487	0.8266
AB1	0.0053	130.7825	0.9891	AB1	0.0017	407.7336	0.9928
AB11	0.0036	192.5409	0.5615	AB11	0.0046	150.6842	0.8631
AB16	0.0027	256.7212	0.6173	AB16	0.0238	29.12383	0.7028
AB18	0.0075	92.41962	0.7002	AB18	0.004	173.2868	0.4408
AB2	0.004	173.2868	0.9231	AB2	0.0053	130.7825	0.8207
AB4	0.0082	84.53014	0.8176	AB4	0.0044	157.5335	0.8001
AB7	0.013	53.31901	0.9337	AB7	0.0078	88.86502	0.5844
BC5	0.0068	101.9334	0.6162	BC5	0.0538	12.88378	0.7952
CE10	0.0047	147.4781	0.828	CE10	0.0011	630.1338	0.5326
CE11	0.0098	70.7293	0.8073	CE11	0.0015	462.0981	0.8242
CE15	0.0145	47.80325	0.839	CE15	0.0032	216.6085	0.7068
CE16	0.0062	111.7979	0.78	CE16	0.0077	90.01911	0.7832
CE2	0.0223	31.08283	0.8646	CE2	0.0064	108.3042	0.86
CE3	0.01	69.31472	0.9494	CE3	0.0052	133.2975	0.9643
CE4	0.0093	74.53195	0.7904	CE4	0.0011	630.1338	0.8701
CE5	0.0014	495.1051	0.6426	CE5	0.0042	165.035	0.972
CE6A	0.0038	182.4072	0.7998	CE6A	0.0017	407.7336	0.9966

Table 3. *Cont.*

Starch				Lignin			
Strain	**Growth Rate (μ)**	**Doubling Time (min)**	**R^2**	**Strain**	**Growth Rate (μ)**	**Doubling Time (min)**	**R^2**
CE7	0.0025	277.2589	0.8929	CE7	0.0077	90.01911	0.5235
CE8	0.0062	111.7979	0.997	CE8	0.0042	165.035	0.6056
CE9	0.0108	64.18029	0.9912	CE9	0.0033	210.0446	0.6926
CEX5	0.0066	105.0223	0.7638	CEX5	0.0034	203.8668	0.8187
CF1	0.0031	223.5959	0.9907	CF1	0.0125	55.45177	0.7057
CF13	0.0076	91.20358	0.9516	CF13	0.0046	150.6842	0.9648
CF14	0.0053	130.7825	0.1813	CF14	0.0036	192.5409	0.6019
CF16	0.0026	266.5951	0.7536	CF16	0.0035	198.0421	0.9336
CF5	0.0029	239.0163	0.7325	CF5	0.0158	43.87007	0.7799
CF7A	0.0018	385.0818	0.8547	CF7A	0.0123	56.35343	0.8978
CF7B	0.0014	495.1051	0.5178	CF7B	0.0036	192.5409	0.8947
CF8	0.0032	216.6085	0.9119	CF8	0.0093	74.53195	0.8128
CFM4A	0.0043	161.197	0.9572	CFM4A	0.0037	187.3371	0.4752
CFM4B	0.0034	203.8668	0.8847	CFM4B	0.0022	315.0669	0.8039
DG1	0.004	173.2868	0.8421	DG1	0.0083	83.51171	0.6603
DG12	0.0076	91.20358	0.6823	DG12	0.003	231.0491	0.6246
DG13	0.0033	210.0446	0.9287	DG13	0.0085	81.54673	0.8483
DG15	0.0203	34.14518	0.8143	DG15	0.0051	135.9112	0.8992
DG16	0.0064	108.3042	0.6816	DG16	0.0016	433.217	0.3942
DG18	0.002	346.5736	0.9231	DG18	0.0092	75.34208	0.9148
DG20	0.0043	161.197	0.9198	DG20	0.0027	256.7212	0.8144
DG21	0.0077	90.01911	0.9434	DG21	0.0133	52.11633	0.9905
DG3	0.0006	1155.245	0.89	DG3	0.0022	315.0669	0.5738
DG5	0.0049	141.4586	0.63	DG5	0.0042	165.035	0.784
DG6	0.0115	60.27367	0.9083	DG6	0.0116	59.75407	0.8613
DGM1	0.0037	187.3371	0.7002	DGM1	0.0103	67.29584	0.8878
DH13	0.0112	61.88814	0.9173	DH13	0.0089	77.88171	0.9231
DH15	0.0051	135.9112	0.9261	DH15	0.0043	161.197	0.6154
DH18	0.0025	277.2589	0.8929	DH18	0.0078	88.86502	0.5787
DH2	0.0021	330.0701	0.9303	DH2	0.0043	161.197	0.6789
DH23	0.0103	67.29584	0.9842	DH23	0.0068	101.9334	0.7872
DH28	0.014	49.51051	0.7901	DH28	0.0032	216.6085	0.7977
DH29	0.005	138.6294	0.9328	DH29	0.0039	177.73	0.6145
DH3	0.0221	31.36413	0.8494	DH3	0.0042	165.035	0.9012
DH31	0.0533	13.00464	0.9603	DH31	0.0058	119.5081	0.9878
DH8	0.0068	101.9334	0.8647	DH8	0.0057	121.6048	0.8454
DH9	0.0046	150.6842	0.7145	DH9	0.0058	119.5081	0.8814

In total, 61 of 124 isolates showed a positive fit and indicated the growth of bacteria. DG6 had a maximum growth rate with both lignin and starch and a doubling time of 60 min, with R^2 values of 0.9083 and 0.8613. A8 showed a high growth rate in both media with R^2 values of 0.8266 and 0.9046 in lignin and starch, respectively, whereas DH13 reflected high R^2 values of 0.9231 and 0.9173. There were some isolates that showed better growth rates in one medium than in the another. For instance, CE11 and CE3 had doubling times of 70 and 69 min in starch but 133 and 462 min in lignin, respectively. The doubling time for BC5 was 13 min in lignin and 102 min in starch media.

From the isolates that were studied for their ability to grow in both substrates, a total of eleven isolates were chosen for further studies, as they had strong growth rates and doubling times in both media. Sites A and D had five and four isolates, respectively, while site C did not have any isolates and site B had only two isolates. These results are in agreement with a metagenomic study of this landfill site that showed that site D was the most diverse in terms of identified bacteria [54]. These isolates were chosen for further assay studies and molecular characterization.

3.3. Enzyme Assays

The hydrolytic abilities of the bacterial isolates are given in Table 4. The result of the qualitative test on proteolytic bacteria in skim milk medium showed that out of the 11 bacteria isolates, 5 bacteria showed clear zones and 6 did not. In total, five isolates, i.e., A19, A6, AB7, DG6 and DH13, were positive for proteolytic activity. In the test for amylase-positive and -negative species, (++) was used to connote a strong positive reaction for starch hydrolysis, (+) for a positive reaction for starch hydrolysis, and (−) for a negative reaction for amylase hydrolysis.

Table 4. Results of hydrolytic assays of bacterial isolates.

Isolate	Amylase	Cellulase	Xylanase	Protease
A3	++	+	+	+
A4	+	−	−	−
A6	+	−	−	+
A8	+	−	−	−
DH31	++	+++	+++	−
AB7	+	+	+	+
BC5	+	+	+	−
DG6	+	++	++	+
DG21	+++	−	−	−
BD25	−	+++	+++	−
DH13	+	+++	+++	+

BD25 is the only isolate that had a negative reaction; DH13, DG6, BC5, AB7, A4, A6 and A8 all had positive reactions, while A19, DH31 and DG21 had strong positive reactions, with the maximum reaction occurring in DG21. In terms of cellulase and xylanase activity, seven showed positive reactions and four showed no reaction at all. Bacterial isolates such as A19, AB7, BC5 and DG5 showed the lowest reactions, DG6 had a slightly higher reaction compared to these four, but DH31, BD25 and DH13 showed highest reactions. The results were the same for both cellulase and xylanase.

The cellulase enzyme activities for the four selected isolates, DH13, DG6, AB7 and A19, were 0.876, 0.931, 1.345 and 1.768, respectively. Based on the results, A19 showed the highest activity, while DH13 showed the lowest. These results are similar to a study conducted by Guder and Krishna [55] in 2019, where cellulase enzymes ranged from 0.119 to 1.6. The authors also concluded that cellulase activity is dependent on the bacterial species.

3.4. Molecular Characterization and Phylogenetic Analysis

Phylogenetic analysis was performed using the results obtained from the 16S rDNA sequencing to allow for the proper identification of promising bacteria due to their enzymatic abilities, as described in this study. The analysis was performed in this way so that the bacteria could be matched to those already existing in the Genbank and to allow them to be maximally identified.

The results show that *Bacillus* strains were the most promising strains identified in this study, and the phylogenetic trees are reflected in Figure 4 and Table 5. *Bacillus* has been identified as an organism capable of withstanding environmental stress and has the simplest nutritional requirements for growth. It is thermophilic in nature and is known to produce hydrolytic enzymes such as α-amylase and protease [56]. The strains identified in the present study include *Bacillus proteolyticus*, *Bacillus Sanguinis*, *Bacillus spizizenii Bacillus paramycoides*, *Bacillus paranthracis and Neobacillus fumarioli.*

Neobacillus fumarioli, formerly *Bacillus fumarioli*, is a thermophilic and aerobic endospore-forming bacteria [57]. *Bacillus paranthracis and Bacillus paramycoides* exhibit bactericidal properties and are used in the mitigation of drought problems [58]. *Bacillus proteolyticus* produces protease and has been used in bioremediation and as a probiotic agent [59]. Three isolates, A6, BD25 and DG21, were identified as *Bacillus paramycoides*. They showed various

responses, with DG21 having higher expression of hydrolytic activities. Our findings that show the strongest positive reaction occurring for amylase enzymes agree with [60], where a bacterial isolate was optimized for amylase production and was identified as *B. paramycoides*.

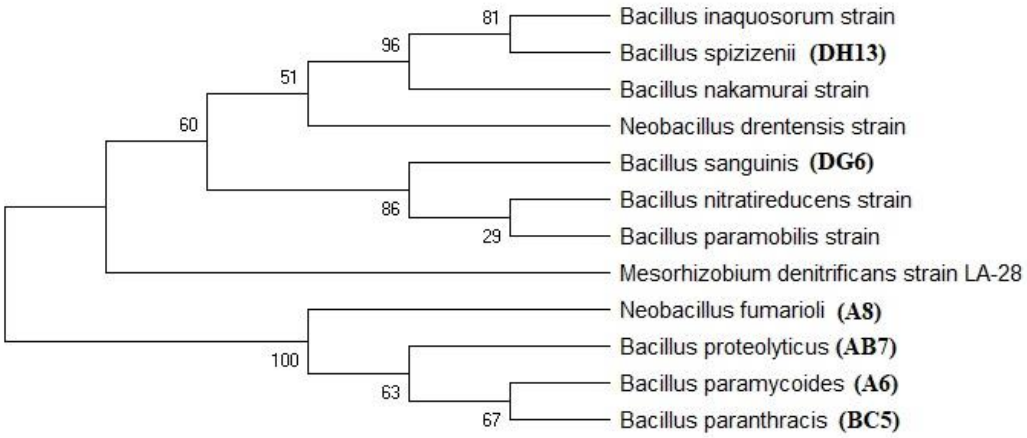

Figure 4. Phylogenetic analysis based on partial 16S rRNA sequences of the bacterial isolates and related *Bacillus* spp. The tree reconstruction was generated using the Neighbor-Joining method.

Table 5. Culture-based and molecular identification of *Bacillus* strains using 16S rRNA gene sequences obtained from waste samples.

Sample ID	Coverage	Similarity	BP	Accession	Matched Bacteria from NCBI
A6	98	99.91	1160	OQ288926	*Bacillus paramycoides*
DG6	96	98.81	2322	OQ288927	*Bacillus Sanguinis*
A8	91	97.11	1270	OQ288921	*Neobacillus fumarioli*
DG21	92	99.75	1315	OQ288922	*Bacillus paramycoides*
DH13	97	98.96	1277	OQ288871	*Bacillus spizizenii*
AB7	97	98.78	1185	OQ288869	*Bacillus proteolyticus*
BC5	95	98.92	1260	OQ288870	*Bacillus paranthracis*

Bacillus species are known to form endospores, which means they are able to endure extreme conditions in their environment. *Acinetobacter*, *Clostridium*, *Bacillus*, *Pseudomonas*, *Desulfuromonas*, *Prevotella*, *Flavobacterium cytophaga*, *Staphylococcus* and *Streptococcus* were reported to be present at a municipal waste landfill site in Poland.

Bacillus species showed an ability for soil decontamination and possible use as an eco-friendly bio-fertilizer to increase crop productivity [61]. *Bacillus species* also produce various metabolites that range from hydrolytic enzymes to bio-pesticides and antibiotics [62]. This ability of *Bacillus* species to secrete extracellular proteins makes them desirable for use in food and drug production [63]. To the best of our knowledge, no studies have reported the presence of *Bacillus spizizenii* or *Neobacillus fumarioli* in landfill or their lignocellulolytic potential. However, other Bacillus strains have been reported to have cellulolytic abilities [26,32].

4. Conclusions

In the present study, we screened and isolated bacteria with enzymatic abilities from sediments. The seven most promising isolates were *Bacillus* species that were grown in both lignin and starch. The results showed that landfill bacteria, such as *Bacillus proteolyticus*, *Bacillus Sanguinis*, *Bacillus spizizenii*, *Bacillus paramycoides*, *Bacillus paranthracis and Neobacillus fumarioli*, were capable of multi-enzymatic activity, as confirmed by 16S rRNA sequencing. The screening of ligninolytic and hydrolytic bacteria may be a key to overcoming challenges in the adoption of lignocellulose as a raw material for bioprocesses. These

bacteria are capable of inducing responses from multiple lignocellulolytic enzymes, and further investigations are needed to determine how they can be adopted in bio-refinery.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9030298/s1, Table S1:title; Bacterial isolates showing results of Gram staining, appearance and growth in lignin and starch media.

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