Isolation and screening of thermophilic *Bacilli* from compost for electrotransformation and fermentation: Characterization of *Bacillus smithii* ET 138 as a new biocatalyst

Elleke F. Bosma¹, Antonius H.P. van de Weijer¹, Martinus J.A. Daas¹, John van der Oost¹, Willem M. de Vos¹ and Richard van Kranenburg¹b.#

Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands¹; Corbion, Gorinchem, The Netherlandsb.

Running head: Screening of thermophilic *Bacilli*

#Address correspondence to Richard van Kranenburg, r.van.kranenburg@corbion.com

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ABSTRACT

Thermophilic bacteria are regarded as attractive production organisms for cost-efficient conversion of renewable resources to green chemicals, but their genetic accessibility is a major bottleneck in developing them into versatile platform organisms. In this study, we aimed to isolate thermophilic, facultatively anaerobic Bacilli that are genetically accessible and have potential as platform organisms. From compost, we isolated 267 strains that produced acids from C5 and C6 sugars at temperatures of 55°C or 65°C. Subsequently, 44 strains that showed the highest production of acids were screened for genetic accessibility by electroporation. Two Geobacillus thermodenitrificans isolates and one Bacillus smithii isolate were found to be transformable with plasmid pNW33n. Of these, B. smithii ET 138 was the best-performing strain in lab-scale fermentations and was capable of producing organic acids from glucose as well as from xylose. It is an acidotolerant strain able to produce organic acids until a lower limit of approximately pH 4.5. As genetic accessibility of B. smithii had not been described previously, six other B. smithii strains from the DSMZ culture collection were tested for electroporation efficiencies and we found the type strain DSM 4216T and strain DSM 460 to be transformable. The transformation protocol for B. smithii isolate ET 138 was optimized to obtain approximately 5x10^3 colonies per µg plasmid pNW33n. Genetic accessibility combined with robust acid production capacities on C5 and C6 sugars at a relatively broad pH range make B. smithii ET 138 an attractive biocatalyst for production of lactic acid and, potentially, other green chemicals.

KEY WORDS

thermophilic Bacilli, electroporation, transformation, isolation, strain selection, biotechnology, green chemicals, Bacillus smithii, genetic accessibility, compost, screening, organic acids
INTRODUCTION

Green chemicals are sustainable bio-based alternatives for chemicals based on fossil resources. Biomass is considered an attractive renewable resource for the production of such green chemicals. Major challenges for using biomass are to develop cost-effective production processes and to convert substrates that go beyond first-generation pure sugars. In this perspective, the use of microbial fermentation processes that convert lignocellulosic sugars is gaining considerable attention. For particular products natural producers can be used, but often *E. coli* and *S. cerevisiae* are used as platform organisms, because of their well-known physiology and ease of engineering for the production of many different products (1, 2).

In general, most organisms used for the production of green chemicals and fuels are mesophiles and current processes are still mainly based on first generation feedstocks, *i.e.* sucrose from sugar beet or sugarcane or glucose derived from starches from corn or tapioca. Although engineering of platform organisms broadens the spectrum of possible substrates (3), the efficiency of the process could be increased by the use of organisms naturally capable of degrading lignocellulose-derived sugars (4). To further increase the efficiency and reduce the costs of the microbial production of green chemicals, moderately thermophilic hosts offer several advantages as compared to mesophilic hosts, including i) reduced cooling costs, ii) lower contamination risk, iii) increased substrate and product solubility and iv) temperature optima of these bacteria matching those of enzymes used for simultaneous saccharification and fermentation (SSF), lowering the enzyme load and costs (5). The use of anaerobic or facultatively anaerobic bacteria eliminates the need for expensive aerated industrial reactors. For SSF, facultative anaerobes have the advantage over strict anaerobes that reduction of the
medium is not necessary, as reduced medium has been shown to inhibit saccharolytic enzymes (6).

Altogether, organisms of particular interest for the production of green chemicals from lignocellulose in an SSF setting are (facultatively) anaerobic thermophiles that can grow in minimal medium and ferment a wide range of substrates including C5 and C6 sugars. They can potentially be used as platform organisms if they are genetically accessible. A number of facultatively anaerobic thermophilic hosts has been studied for green chemical and fuel production (reviewed in (7, 8)), such as Bacillus coagulans for lactic acid (9, 10), Bacillus licheniformis for 2,3-butanediol (11, 12) and Geobacillus thermoglucosidans for ethanol production (13). Examples of strictly anaerobic thermophiles that have been studied for biofuel production are Clostridium thermocellum (14, 15), several Caldicellulosiruptor spp. (16-18) as well as Thermoaerobacterium spp. and Thermoanaerobacter spp. (18-21). For each of these species, one or more strains have been shown to be genetically accessible and engineering tools have been developed (7, 22). Still, it is desirable to further develop more efficient production organisms and improved genetic tools. On the one hand this will enable a better understanding and exploitation of the diversity in metabolism found in thermophilic organisms and on the other hand this will expand the genetic toolbox for thermophiles, which at the moment is still rather limited compared to those available for mesophilic platform organisms.

In this study we aimed to extend the collection of potential industrial platform organisms by isolating thermophilic, facultatively anaerobic Bacilli and selecting a suitable production host by screening for C5 and C6 sugar utilization, organic acid production, fermentation performance and genetic accessibility in order to select a new biocatalyst that has the potential to be developed into a platform organism.
MATERIALS AND METHODS

Media, cultivation methods and strains

Thermophile Minimal Medium (TMM, adjusted from (23)) contained per L: 8.37 g MOPS and 100 mL 10x concentrated Salt Solution (TSS-10x, containing per L: 2.3 g K2HPO4; 5.1 g NH4Cl; 50 g NaCl; 14.7 g Na2SO4; 0.8 g NaHCO3; 2.5 g KCl; 18.7 g MgCl2.6H2O; 4.1 g CaCl2.2H2O; 0.08 g SrCl2.6H2O; 0.08 g H3BO3; 9.0 g NaNO3). The pH was set at 6.94 at room temperature unless indicated otherwise, the medium was autoclaved for 20 min at 121°C and cooled to max. 80°C, after which substrate and 10 mL per L of 1 mM FeSO4.7H2O in 0.4 M tricine was added.

TMMY is TMM supplemented with 0.5 g/L yeast extract (Roth, Germany).

Thermophile Vitamin Medium with Yeast extract (TVMY) resembles TMMY, with the exception that SrCl2.6H2O, H3BO3 and NaNO3 is omitted and that metal and vitamin mixtures are added after sterilization. Metal mix (1000x concentrated) contained per L: 16.0 g MnCl2.6H2O; 1.0 g ZnSO4; 2.0 g H3BO3; 0.1 g CuSO4.5H2O; 0.1 g Na2MoO4.2H2O; 1.0 g CoCl2.6H2O; 7.0 g FeSO4.7H2O. Vitamin mix (1000x concentrated) contained per L: 0.1 g thiamine; 0.1 g riboflavin; 0.5 g nicotinic acid; 0.1 g panthothenic acid; 0.5 g pyridoxamine, HCl; 0.5 g pyridoxal, HCl; 0.1 g D-biotin; 0.1 g folic acid; 0.1 g p-aminobenzoic acid; 0.1 g cobalamin.

LB2 contained per L: 10 g tryptone (Oxoid, United Kingdom), 5 g yeast extract (Roth), 100 mL TSS-10x. pH was set to 6.94 – 7.00 at room temperature.

TGP2 (adjusted from (24)) contained per L: 17 g tryptone (Oxoid), 3 g neutralized soy peptone (Oxoid), 100 mL TSS-10x. pH was set to 6.94 – 7.00 at room temperature. After
autoclaving, 4 mL/L sterile 60% glycerol and 4 g/L filter sterilized 200 g/L sodium pyruvate were added.

For all tube and plate cultures, carbon substrates were used at a concentration of 10 g/L. For cultivation in fermenters, carbon substrates were used in a concentration of 30 g/L unless indicated otherwise. Substrates were added separately as 50% sterile solutions after autoclaving of the medium; xylose and arabinose were filter sterilized and glucose and sucrose were autoclaved. For plates, 5 g/L gelrite (Roth) was added. The acid indicator bromocresol purple was used to monitor acidification in plates and tubes. Anaerobic cultivation of plates was performed in an anaerobic jar (Oxoid) containing a GasPak (Oxoid). All strains were routinely grown at their isolation temperature (55°C or 65°C), unless stated otherwise. All strains obtained from DSMZ were routinely grown at 55°C.

To evaluate the optimum temperature of strain ET 138, cells were grown overnight from glycerol stock in 10 mL LB2 at 55°C and next morning diluted to an OD₆₀₀ of 0.05 in 20 mL TMMY supplemented with 10 g/L glucose in 50 mL Greiner tubes and incubated in water baths at different temperatures without shaking. A second experiment was performed in the same way, but with the overnight cultures grown at the same temperatures as to which they were transferred next morning. Tested temperatures were 37, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 57, 60, 63, 66 and 69°C. All these experiments were performed in triplicate. The same experimental set-up was used to evaluate the optimum pH of ET 138, but then the temperature was set at 55°C and the initial pH of the medium was varied from 3.0 to 7.0 with intervals of 0.5. For pH values from 3.0-5.5, MES buffer was used instead of MOPS.

The following strains were obtained from DSMZ (Germany): *G. thermoglucosidans* DSM 2542ᵀ, *B. coagulans* DSM 1ᵀ, *B. smithii* DSM 4216ᵀ, *B. smithii* DSM 459, *B. smithii* DSM 460, *B. smithii* DSM 2319, *B. smithii* DSM 2320 and *B. smithii* DSM 2321.
Sampling, isolation and initial selection

Fresh compost samples were collected from two compost heaps at Recom Ede (NL) on August 5, 2010, both consisting solely of plant material. One heap was sieved through a 2-3 cm mesh and the other through a 1-2 cm mesh. The heaps are mixed every two weeks, resulting in an environment suitable for isolating facultatively anaerobic organisms. The temperature of the compost was 75°C at ±30 cm depth and sampling material was taken from the surface until ±30 cm depth. Samples were taken by scraping compost into a plastic jar, transported to the laboratory (±20 min) at ambient temperature to the lab and stored at 4°C until use (1-4 weeks).

Five different conditions were applied during the isolation procedure, namely: 1: sucrose at 55°C, 2: sucrose at 65°C supplemented with 5 g/L CaCO₃, 3: sucrose at 55°C supplemented with 5 g/L CaCO₃, 4: glucose at 65°C supplemented with 5 g/L CaCO₃, 5: glucose at 55°C supplemented with 5 g/L CaCO₃. All conditions were in TMM medium containing the indicated carbon source and were kept the same throughout the whole procedure for each condition. The isolation procedure was as follows: for each of the two compost types, 5 g compost was added to a 250 mL Erlenmeyer flask containing 50 mL TMM medium supplemented with the indicated carbon source and shaken for 6 h at 120 rpm at either 55°C or 65°C. After 6 h, dilution series were plated on the same medium containing 5 g/L GelRite (Roth) and grown anaerobically for 24-48 h at either 55°C or 65°C. Subsequently, single colonies were picked and each colony was streaked to 4 plates, containing TMM with either glucose, xylose, arabinose or sucrose and incubated anaerobically for 24-48 h at the same temperature. Strains grown on 3 or more sugars, of which at least one C5 and one C6 sugar, were inoculated into 8 mL of their isolation medium containing bromocresol purple in 15 mL screw-capped tubes and incubated without shaking for 24-48 h at 55 or 65°C. For the
In order to perform a production-based screening, all isolated strains were inoculated in duplicate from glycerol stocks into 8 mL TMMY supplemented with 10 g/L glucose and 500 µL of 80% CaCO₃ in 15 mL tubes, placed horizontally while shaking at 40 rpm and incubated for 48 h at their isolation temperature (55°C or 65°C), after which OD₆₀₀ and pH were measured and HPLC analysis was performed. Based on HPLC results, pure cultures were made for selected strains by transferring single colonies twice on TMMY plates supplemented with 10 g/L glucose before finally transferring them to the same medium in liquid form to make stocks after 24-30h of growth. Pure cultures were subjected to 16S identification and used for further studies.

16S rRNA identification and phylogenetic analysis

A little material from single colonies was transferred from plates to a PCR tube using a sterile toothpick and subsequently incubated in a microwave for 1.5 min at 800W. PCR mix was added containing 2.5 U DreamTaq DNA polymerase (Fermentas, United Kingdom), DreamTaq buffer (Fermentas), 1 mM dNTPs (Fermentas), 0.2 µM primers GM3 (AGAGTTTGATCATGGC) and GM4 (TACCTTGTTACGACTT) and MilliQ water to a total volume of 50 µL. PCR products were checked on agarose gel. Products were purified either by GeneJet PCR purification kit (Fermentas), GeneJet Gel Extraction kit (Fermentas) or by a sequencing company, being either BaseClear (NL) or GATC (DE). GM3 and GM4 sequences were assembled to one sequence using CloneManager and manually curated and trimmed until 1358 bp, after which BLASTn was used for identification against the 16S database. Mega6 software (25) was used for creating the alignment using Clustal (26) after which the
Neighbour Joining method (27) was used to create the phylogram and bootstrap analysis (28) was performed using 1000 replicate analyses.

**Testing genetic accessibility**

Depending on their 16S rRNA gene-based genus identification, strains selected in the initial selection were subjected to a transformation protocol for either *Geobacillus* (24) or *Bacillus* (29) with slight modifications as follows: strains were grown overnight at their isolation temperature in 10 mL medium in a 50 mL Greiner tube and next morning, cultures were diluted to an OD$_{600}$ of 0.08 in 100 mL medium for *Bacilli* and 50 mL medium for *Geobacilli*. For strains belonging to the genus *Bacillus*, LB2 medium was used and for strains belonging to the *Geobacillus* genus, TGP2 medium was used, with the exception of *Geobacillus* strains that grew better on LB2 (isolates ET 036, ET 050, ET 130, ET 144-2 and ET 261-3). Cultures were grown until they reached an OD$_{600}$ of 0.45-0.65 for *Bacilli*, and around 1.00 for *Geobacilli*. After washing 3x with SG buffer for *Bacilli* (per L: 171.2 g sucrose, 0.2 g MgCl$_2$, 50 mL glycerol) or 4x with electroporation buffer for *Geobacilli* (per L: 91 g sorbitol, 91 g mannitol, 100 mL glycerol), final pellets were resuspended in 240 µL SG buffer for *Bacilli*, and 900 µL electroporation buffer for *Geobacilli*, after which cells were either stored at -80°C or electroporation was performed directly. Electroporation settings were 2.0 kV, 25 µF, 200 Ω in a 2 mm cuvette (based on (30)) for all strains. Additional settings were tested as follows: 1.5 kV, 25 µF, 600 Ω in a 1 mm cuvette (29) for *Bacilli* strains ET 138, ET 143, ET 224, ET 226, ET 229, ET 230-1, ET 239-2, ET 244, ET 261-1 and ET 263; 2.5 kV, 25 µF, 600 Ω in a 1 mm cuvette (24) for *Geobacilli* strains ET 006, ET 011-1, ET 036, ET 042, ET 050 and ET 208; 2.5 kV, 25 µF, 600 Ω in a 2 mm cuvette (based on (24)) for ET 006, ET 036, ET 042, ET 050, ET 072, ET 129 and ET 208. Recovery was performed at 3°C below the isolation temperatures for 3 h in RG2 medium (LB2 with 121 g/L sucrose and 10 g/L
glucose) for Bacilli and for 2h in LB2 or TGP2 supplemented with 0.5% (w/v) glucose for
Geobacilli. G. thermoglucosidans DSM 2542$^T$ and B. coagulans DSM 1$^T$ were used as
positive controls. All strains were tested with plasmid pNW33n (Bacillus Genetic Stock
Centre) and 7 µg/mL chloramphenicol was added to the plates after transformation. Negative
controls were performed by electroporating without adding any plasmid DNA.

Colonies appearing after transformation were streaked to a new plate and grown
overnight, after which grown streaks were subjected to colony PCR as described above, using
primers BG3464 (AACTCTCCGTGCCTATTGTAACCA) and BG3465
(TATGCGTGCAACGGAAGTGAC). To confirm transformation, positive colonies were
inoculated into 10 mL LB2 or TGP2 supplemented with 7 µg/mL chloramphenicol in a 50 mL
tube and grown overnight at their isolation temperature. From this culture, plasmid was
extracted using the GeneJET Plasmid Miniprep Kit (Fermentas) with 4 mg/mL lysozyme
(Sigma, USA) added to the resuspension buffer, in which resuspended pellets were incubated
for 30 min at 37°C. Extracted plasmid was used for PCR as described above and restriction
analysis using 550-650 ng plasmid DNA with Fermentas enzymes StuI and HindIII for 2 h at
37°C. Plasmid extraction on non-transformed strains was used as negative control. In case of
inconclusive band patterns, the isolated plasmid DNA was transformed to E. coli DH5α and
subsequently re-isolated and digested.

**Fermentations**

Pre-cultures of transformable isolates were grown overnight from glycerol stocks in 10
ml LB2 at 55°C in a 50 mL Greiner tube without shaking. Next morning, 10 mL culture was
transferred to 40 mL TMMY medium supplemented with 30 g/L glucose in a 250 mL
Erlenmeyer flask and incubated at 55°C without shaking. When the cultures reached an OD$_{600}$
of 0.3-0.7 (exponential growth phase), 20 mL was used to inoculate a reactor containing 1 L
TMMY medium without MOPS and supplemented with 30 g/L glucose. Glass reactors of 2 L working volume were used (Applikon, The Netherlands) under control of an ADI 1010 Bio-controller (Applikon) with an ADI 1025 Bio-console. Temperature was controlled at 55°C, stirring speed was 150 rpm and pH was maintained at 6.5 unless stated otherwise by addition of 3 M KOH. Growth was monitored off-line by absorbance at 600 nm (OD$_{600}$) and sugar and fermentation products were measured by high-pressure liquid chromatography (HPLC).

Fermentations of ET 138 at different pHs were performed in the same way as described above, but overnight cultures were transferred to TMMY medium with the same pH as the reactor (either 4.5, 5.5 or 6.5), and pH of the reactors were controlled at pH 4.5, 5.5 or 6.5.

For the comparison of ET 138 on glucose and xylose, pre-cultures were inoculated from glycerol stock and grown overnight at 55°C in 10 mL TVMY medium supplemented with either 10 g/L glucose or 10 g/L xylose in a 50 mL tube at 150 rpm. Next morning, 1 mL culture was transferred to 50 mL of the same medium in a 250 mL Erlenmeyer flask at 55°C and 150 rpm. When this culture reached an OD$_{600}$ of 0.5-0.7, 20 mL was used to inoculate a reactor. In the reactor, 1 L TVMY medium without MOPS was used, supplemented with 20 or 25 g/L xylose or glucose, as indicated.

**Analytical techniques**

Sugars and fermentation products were quantified using an HPLC system from Thermo containing a P2000 pump, an AS3000 autosampler, a UV/VIS1000 detector and an RI-150 refraction index detector. A Shodex RSpak KC-811 cation-exchange column was used with a mobile phase of 3 mM H$_2$SO$_4$ and operated at 0.8 mL/min and 80°C. All samples were diluted 1 on 1 with 10 mM DMSO in 0.04 N H$_2$SO$_4$. D-lactate and L-lactate were distinguished via enzymatic assay kits (MegaZyme D-LATE and K-DATE).
Unknown peaks observed in the HPLC spectrum in the xylose fermentation were collected from HPLC, freeze-dried and analyzed by NMR by Biqualys (NL). NMR-identification was confirmed by running the corresponding standards on HPLC.

**GenBank accession numbers**

All the 16S rRNA gene sequences generated in this study have been submitted to the GenBank database with accession numbers KP010222-KP010265.

Type strains used for the phylogenetic tree were trimmed to the same 1358 bp as the isolates and were derived from the following GenBank accession numbers: NR_043021.1 (G. thermodenitrificans DSM 465), NR_036987.1 (B. smithii DSM 4216), NR_109664.1 (B. thermocopiae SgZ7), NR_115727.1 (B. coagulans DSM 1), NR_026515.1 (A. pallidus DSM 3670), NR_043022.1 (G. thermoglucosidans DSM 2542), NR_028708.1 (G. caldoxylosilyticus DSM 12041).
RESULTS

Isolation, initial selection and identification. Thermophiles able to grow on minimal medium (TMM) at 55°C or 65°C were obtained from compost (Table 1). Single colonies (842) from the dilution series of compost samples were transferred to plates containing different carbon sources (glucose, sucrose, xylose, or arabinose). All colonies grew on glucose, and 584 colonies also grew on one or both of the C5 sugars xylose and arabinose at 55°C or 65°C. These 584 colonies were then inoculated into liquid TMM supplemented with a pH indicator under microaerobic conditions. Glycerol stocks were made of 267 strains that showed both growth and acidification in liquid culture on glucose (Table 1). To select the best acid-producing strains, all 267 isolates were inoculated in duplicate in TMM liquid medium supplemented with glucose, CaCO₃ and 0.5 g/L yeast extract and grown for 48 h under microaerobic conditions, after which HPLC analysis was performed. For each isolation condition, strains were found that either produced relatively high quantities of total products, or showed products that were rarely found among strains isolated under the same conditions. A total of 35 strains was selected from the five different isolation conditions (Table 1 and S1) to make pure cultures to screen for genetic accessibility. For ten isolates (ET 011, ET 072, ET 129, ET 130, ET 131, ET 144, ET 230, ET 239, ET 261, ET 267), purification revealed distinct morphologies and resulted in more than one pure culture from the same original colony (e.g. 144-1 and 144-2). The 44 pure cultures were identified by 16S rRNA gene sequence analysis (Figure 1). The majority of strains was identified as *Geobacillus thermodenitrificans*, followed by *Bacillus thermocoprae*, *Geobacillus caldoxyllosilyticus*, *Bacillus coagulans* and *Aeribacillus pallidus*. One strain of *Bacillus smithii* was obtained. The highest species diversity was obtained with isolation condition 5, whereas this was the lowest in conditions 1 and 4, in which mainly *G. thermodenitrificans* were found. As expected, at
65°C mainly Geobacillus species were obtained, whereas at 55°C also Bacillus species were found and the species diversity was larger.

HPLC data for the tube test for the selected strains are shown in Table S1. Independent of the species, all strains produced mainly lactate, with acetate as the major by-product and succinate as a minor by-product. Gas production was not analyzed. All strains, except for Bacillus smithii, also produced small amounts of one or more other minor products such as ethanol, 2,3-butanediol (2,3-BDO), formate, propionate or malate. By-products varied not only between species, but also between strains of the same species. For example, Geobacillus thermodenitrificans ET 236 and ET 251 produced malate, ethanol, formate and 2,3-BDO, whereas G. thermodenitrificans ET 136 did not produce detectable amounts of malate and 2,3-BDO, but higher amounts of ethanol, and G. thermodenitrificans ET 241 did not produce any ethanol. Most B. thermocopriae strains produced propionate. The addition of CaCO₃ in the tubes might have enhanced production of C4-dicarboxylic acids, but these were never observed as major products. The highest titer of these products was reached by strain G. caldoxylosilyticus ET 208, with 0.58 g/L succinate and 0.18 g/L malate, together constituting 15% of its total production, with its main products being lactate and acetate. The total amount of products varied strongly from 1.27 g/L to 7.61 g/L. On average, strains isolated in condition 5 (glucose 55°C with CaCO₃) produced most acids, while strains from condition 2 (sucrose 65°C) produced the least and strains isolated at 55°C on average produced more acid compared to the 65°C-isolates. No difference was observed in average production between strains isolated on sucrose and glucose.

**Screening for genetic accessibility by electrotransformation.** The 44 selected pure cultures were tested for their transformation efficiency by electroporation. Depending on the species,
slightly modified versions of either a *Bacillus* (29) or a *Geobacillus* (24) protocol were used to prepare competent cells. Subsequently, the obtained cell suspensions were electroporated using *E. coli* – (Geo)bacillus shuttle vector pNW33n. Strains that were successfully transformed with pNW33n were *B. smithii* strain ET 138 and *G. thermodenitrificans* strains ET 144-2 and ET 251. Transformation was confirmed by colony PCR and by isolating plasmid DNA from the transformed strains, which was subsequently confirmed to be pNW33n by restriction analysis of the isolated plasmid DNA from the transformants (*G. thermodenitrificans* strains ET 144-2 and ET 251) or from retransformed *E. coli* (*B. smithii* ET 138) (Figure S1). Transformation of *G. thermodenitrificans* strains ET 144-2 and ET 251 according to the *Geobacillus* protocol (with settings 2.0 kV, 25 µF and 200 Ω in a 2 mm cuvette) resulted in 6 and 4 colonies per µg DNA, respectively. *B. smithii* strain ET 138 made competent according to the *Bacillus* protocol and electroporation settings (29) yielded 3 colonies per µg DNA, whereas the *Geobacillus* electroporation settings yielded 33 colonies per µg DNA for this strain. Subsequent retesting of other *Bacillus* isolates with the *Geobacillus* settings did not result in any transformants. For strains ET 144-2 and ET 251, transformation was reproducible but not always successful, whereas transformation for ET 138 was highly reproducible despite the low efficiency. For fourteen strains, occasionally colonies also appeared when no DNA was added during electroporation, while the strains did not grow on chloramphenicol prior to transformation (Table S1). None of the colonies formed by these strains tested positive in the PCR on the pNW33n repB-cat-gene combination. As transformation is crucial for the development of an organism into an efficient platform organism, only the three transformable strains were selected for further studies.
Fermentation and growth characteristics of transformable strains. Transformable isolates *B. smithii* ET 138 and *G. thermodenitrificans* 144-2 and 251 were tested in 1 L pH-controlled fermentations on glucose. Similar to the results of the acidifying tube tests, for all three strains, lactate was the major product and acetate the second most abundant product, with succinate as minor by-product (Table 2). For all three strains, ethanol production was observed in one of the two duplicate fermentations, while this was not observed during the tube experiments for *B. smithii* ET 138 (Table S1). *G. thermodenitrificans* ET 251 showed very low titers and productivity in the reactor, namely a total of 6.58 ±1.35 g/L with 0.05 ±0.01 g/L/h (Table 2), even though it performed well in tubes (Table S1). *G. thermodenitrificans* ET 144-2 produced a total of 14.80 ±4.03 g/L products, but showed a rather low productivity of 0.08 ±0.01 g/L/h (Table 2). In contrast, *B. smithii* ET 138 outperformed the other strains both in product titer and productivity with 19.35 ±2.34 g/L at 0.14 ±0.01 g/L/h (Table 2). As it was also outperforming the other two isolates in transformation efficiency, *B. smithii* ET 138 was selected for further studies.

*B. smithii* ET 138 showed growth at the tested temperatures between 37 and 63°C, with optimal growth between 51 and 57°C and no growth at 66°C or higher. The optimum pH was found to be 6.5, with little difference between pH values of 6.0-7.0 (data not shown). In further experiments, growth conditions were kept at 55°C and pH 6.5. The lowest initial pH value still supporting growth was 4.5. To further evaluate the ability of *B. smithii* ET 138 to ferment at low pH, fermentations in pH-controlled reactors were performed at pH 4.5, 5.5 and 6.5 (Table 3). During the first 43 h of fermentation, product titers and productivities at pH 5.5 and 6.5 were almost identical with 10.3 g/L at 0.24 g/L/h and 10.8 g/L at 0.25 g/L/h, respectively. After 141 h, both titer and productivity were 1.5-fold lower at pH 5.5 compared to pH 6.5 (Table 3). At pH 4.5 the strain was still able to grow and produce for 43 h, but titer
and productivity were reduced to 3.6 g/L and 0.08 g/L/h. Hardly any acid was produced after 43 h, indicating severe acid stress (Table 3). At pH 5.5 and 6.5 the relative product distributions were equal with 96.5% lactate, 3% acetate and 0.5% succinate. At pH 4.5, this changed slightly to 91% lactate, 8% acetate and 1% succinate.

Next, we evaluated the ability of B. smithii ET 138 to ferment xylose (Figure 2 and S2), which is the most abundant sugar in lignocellulose next to glucose. The medium was changed from TMM to TVMY and lower substrate concentrations of 20 g/L were used to decrease browning of the medium with xylose, which was observed when using 30 or 25 g/L xylose, probably due to a Maillard reaction. The growth curve on both substrates was highly similar (Figure 2 and S2). On both glucose and xylose, growth stopped after approximately 24 h while the acid production continued with only a small decrease in productivity, indicating that production is uncoupled from growth. The product profile on both substrates was found to be similar with lactate being the major product, i.e. 91% (13.9 g/L) on xylose and 92% (17.46 g/L) on glucose, with an optical purity of 99% L-lactate. On both sugars, acetate was the major by-product, followed by minor amounts of succinate and malate (Figure 2). In the xylose fermentation, two unknown peaks were observed in the HPLC spectrum, which were analyzed by NMR. One peak was identified as xylitol and was produced in amounts of approximately 1.5 g/L. The other peak was likely to be an acetylated form of xylose but could not be further identified with certainty. Whereas glucose is completely consumed at the end of fermentation, xylose is not. This might be related to browning of the medium after approximately 70 h, which was also observed at 25 g/L xylose concentrations (Figure S2) and was followed by a slight pyruvate accumulation.
Optimization of transformation for *B. smithii* ET 138 and other *B. smithii* strains. The electroporation protocol for *B. smithii* ET 138 was optimized for increased transformation efficiencies. Out of 5 different tested electroporation settings (Table S2), the best was found to be 2.0 kV, 25 μF, 400 Ω in a 2 mm cuvette, resulting in 160 colonies per μg DNA (Table 4). Induction of antibiotic resistance by addition of a sub-lethal (1000x diluted) concentration of antibiotics after 2 h of recovery as described for *B. coagulans* (31) had no effect (Table S2). In contrast, the recovery medium had a large impact. Changing the original RG2 recovery medium (LB2 with 121 g/L sucrose and 10 g/L glucose) to LB2 resulted in an increase in transformation frequencies to ~1000 colonies per μg DNA. Transformation efficiency could be further increased to ~2000 by growing the cells in larger flasks prior to making them competent to allow for more aeration and faster growth (Table 4). Substituting the SG-buffer for the *Geobacillus*-electroporation buffer strongly reduced efficiencies to 40 colonies per μg DNA (Table S2). Lastly, we tested smaller amounts of added plasmid DNA. In all initial experiments, 1.0-2.5 μg DNA was added. Using only 20 ng DNA for transformation of *B. smithii* ET 138 resulted in a maximum efficiency of ~5000 colonies per μg DNA (Table 4). Storage of competent cells at -80°C was tested for periods up to one year and did not affect the transformation efficiency.

We also tested *B. smithii* strains from the DSMZ culture collection for transformation to evaluate their genetic accessibility. Using the optimized protocol for ET 138, a transformation efficiency of 10-100 colonies per μg plasmid DNA was obtained for type strain DSM 4216<sup>T</sup>, which is approximately 20-200 times lower than that for strain ET 138 with the same conditions. Also for DSM 4216<sup>T</sup>, different electroporation conditions and recovery media were tested (data not shown). For DSM 4216<sup>T</sup>, the difference between recovery medium LB2 and RG2 was less pronounced than for ET 138. The best settings for DSM 4216<sup>T</sup>...
were 1.5 kV, 25 µF, 600 Ω in a 1 mm cuvette with recovery for 3 h in LB2, yielding 10-200 colonies per µg plasmid DNA. Under the tested conditions, transformation for the type strain was found to be less reproducible than for isolate ET 138. The optimal settings for type strain DSM 4216T and isolate ET 138 were used to evaluate transformation of 5 more publically available \textit{B. smithii} strains. Of those strains, only DSM 460 generated transformants (25 colonies per µg DNA) but no attempts for further optimization were made. The best and most reproducibly transformable \textit{B. smithii} strain so far is our isolate ET 138 with a maximum of ~5000 colonies per µg DNA.

Similar attempts to increase transformation efficiencies by changing electroporation settings and buffers for ET 144-2 were unsuccessful (data not shown). For strain ET 251, no transformation optimization was performed because this strain showed poor fermentation performance (Table 2).
DISCUSSION

In this study we aimed at isolating thermophilic Bacilli that are genetically accessible and our isolation conditions were aimed at finding industrially relevant strains: minimal medium was used, temperature was set to moderately thermophilic temperatures, both micro-aerobic and anaerobic conditions were applied during the isolation procedure to find facultative anaerobes, and utilization of both C5 and C6 sugars was evaluated. Using 5 different isolation conditions, we isolated 267 strains from compost, of which 44 were identified by 16S rRNA gene sequencing that all belonged to the family Bacillaceae. The observed abundance of the genera Bacillus and Geobacillus (notably G. thermodenitrificans) among the isolates from the thermophilic compost is in line with a recent report describing the microbial succession during thermophilic composting (34). In contrast to Li et al., we did not find any Ureibacilli and B. licheniformis but isolated several B. coagulans strains and one B. smithii (34). These differences might be explained by the fact that for isolation we used a minimal medium and compost that consisted solely of plant material, whereas the other study made use of rich LB medium and compost that contained both plant material and manure (34, 35).

Product formation on glucose was evaluated for all our isolates. In general, the product profiles are in line with other reports on thermophilic Bacilli (23, 33, 35). Whereas these studies mainly focused on a particular species or strain, our data provide a comparison of several thermophilic Bacillus species and strains grown under the same conditions. This revealed homogeneity in the main products lactate and acetate, whereas a large diversity was observed in the formation of by-products between species as well as between strains of the same species. This diversity creates opportunities for the use of these organisms for the production of different chemicals and their use as platform organisms or source of enzymes...
and pathways. G. thermoglucosidans DSM 2542\textsuperscript{T} and B. coagulans DSM 1\textsuperscript{T} were included as benchmarks because these two organisms have been studied for production of ethanol (36) and lactate (31), respectively, and both are genetically accessible (24, 31, 37). B. smithii ET 138 was the only strain from our study that had genetic accessibility and product titers comparable to the benchmark organisms.

To the best of our knowledge, this is the first large-scale screen of genetic accessibility among different species of thermophilic Bacilli. Although several thermophilic Bacillus species have been described to be genetically accessible (11, 24, 29, 31, 38-40), often laborious protocols such as protoplast transformation are required. Moreover, for each species highly variable transformation efficiencies have been reported for a limited number of strains, suggesting that genetic accessibility is very strain-specific. Strain-specificity is also implicated by Patel et al. who reported only one of their B. coagulans isolates to be genetically accessible (33). This is supported by our findings: in the first isolation round, only 2 out of 25 tested G. thermodenitrificans isolates were genetically accessible and no G. stearothermophilus or B. coagulans strains were transformable under the tested conditions. After testing the electrotransformation for all selected isolates, 3 strains were transformable: B. smithii ET 138 and G. thermodenitrificans ET 144-2 and ET 251, of which ET 138 was the most reproducible. A second isolation round, in which we enriched strains in reactors, resulted in four G. thermoglucosidans strains that could be reproducibly transformed and one G. toebii strain that was not reproducibly transformable (data not shown). Further optimization of the protocols per strain or the use of other transformation methods such as protoplast transformation or conjugation might increase the amount of transformable strains, but for high throughput engineering and use as platform organism, electroporation is a fast and convenient method. As B. smithii ET 138 outperformed the other strains in both fermentation and
transformation via simple electroporation, we focused optimization experiments on this strain. The optimized electroporation efficiency of *B. smithii* ET 138 of $5 \times 10^3$ using pNW33n is close to that found in *B. coagulans* P4-102B with $1 \times 10^4$ using pNW33n (29) and *G. thermoglucosidans* with $1 \times 10^4$ using *E. coli-Geobacillus* shuttle vector pUCG18 (24).

*B. smithii* was originally re-classified from *B. coagulans* (41), but contrary to *B. coagulans*, genetic accessibility has not been reported previously. *B. smithii* has been described for the production of several biotechnologically interesting enzymes such as nitrile hydratase (42), endoinulinase (43) and alkaline lipase (44). Its biotechnological potential was further reported in an isolation study targeted at xylose-utilizing, ethanol-tolerant ethanol-producers (45), as well as during lignocellulose-based composting, where *B. smithii* was among three bacterial compost isolates showing ligninolytic activity (46). Several *B. smithii* strains were isolated from a sugar beet factory and shown to harbour glycosylated S-layer proteins and to secrete lactic acid as their main product (47). The sugar beet factory isolates showed temperature curves ranging from 37°C to 65°C, although not all strains grew at 37°C and the type strain did not grow at 65°C. The sugar beet extraction plant that was used as isolation source was around pH 4.5, indicating acid tolerance of the strains (47). In line with these findings, our isolated strain ET 138 grows between 37°C and 65°C, with highly similar growth rates between 51 and 57°C and it is still well able to grow and produce acids at pH 5.5 and even to some extent at pH 4.5. Such robustness against temperature and pH fluctuations is an important advantage in industrial applications to increase process stability. Another useful industrial property is its capacity to uncouple growth and acid production. Its robustness, combined with the ability to efficiently utilize C5 and C6 sugars present in lignocellulosic substrates and being genetic accessible, makes *B. smithii* an attractive biocatalyst for lactic acid production, having the potential to be developed into a novel
platform organism. Genome sequencing is currently on-going, as well as the development of genetic tools to allow its further exploitation by metabolic engineering.

CONCLUSION
Thermophilic Bacilli able to ferment glucose and xylose can readily be isolated from compost. All produce lactic acid and acetic acid under micro-aerobic conditions, while production of other products such as succinate, malate, ethanol, formate, 2,3-butanediol, or propionate is variable. Genetic accessibility via electroporation is scarce and strain-dependent. We have isolated B. smithii ET 138 and established an efficient transformation protocol. The combination of fermentation robustness and genetic accessibility makes this strain an attractive biocatalyst and candidate for further development into a platform organism for production of green chemicals from renewable resources.

CONFLICT OF INTEREST
The authors declare to have no conflict of interest. RvK is employed by the commercial company Corbion (Gorinchem, The Netherlands).

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REFERENCES


isolates with glycosylated surface layer (S-Layer) proteins and their affiliation to

FIGURE LEGENDS

Figure 1. Neighbour-joining tree of 16S rRNA gene sequences of selected isolates and type strains. The first number indicates the isolation round, whereas T indicates a type strain. Numbers at the nodes represent bootstrap values out of 1000 replicates.

Figure 2. Fermentation of B. smithii ET 138 on xylose and glucose. Fermentation was carried out in 1 L TVMY supplemented with 20 g/L xylose or glucose, at 55°C, pH 6.5, 150 rpm and without any gas addition. Grey line with plus-symbol: OD₆₀₀; open circles: xylose or glucose; filled diamonds: lactate; closed squares: acetate; crosses: malate and succinate; closed triangles: pyruvate. During the xylose fermentation, browning of the medium was observed after approximately 70 h.
**TABLE 1** Overview of the number of isolates per step for each isolation condition.

<table>
<thead>
<tr>
<th>Isolation condition</th>
<th>Strain (ET)</th>
<th>Nr of colonies selected from dilution series and plated on 4 sugars</th>
<th>Nr of strains growing on at least 3 sugars and inoculated into liquid medium</th>
<th>Nr of strains acidified and stocked</th>
<th>Nr of strains selected based on HPLC data and purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: TMMscr</td>
<td>65°C</td>
<td>001-054</td>
<td>257</td>
<td>217</td>
<td>53</td>
</tr>
<tr>
<td>2: TMMscr+CaCO₃ 65°C</td>
<td>055-131</td>
<td>260</td>
<td>151</td>
<td>77</td>
<td>5</td>
</tr>
<tr>
<td>3: TMMscr+CaCO₃ 55°C</td>
<td>132-157</td>
<td>65</td>
<td>45</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>4: TMMglc+CaCO₃ 65°C</td>
<td>158-213</td>
<td>130</td>
<td>77</td>
<td>56</td>
<td>5</td>
</tr>
<tr>
<td>5: TMMglc+CaCO₃ 55°C</td>
<td>214-267</td>
<td>130</td>
<td>94</td>
<td>55</td>
<td>13</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>842</td>
<td>584</td>
<td>267</td>
<td>35</td>
</tr>
</tbody>
</table>

*a* Abbreviations: scr: sucrose; glc: glucose.

*b* Tested sugars were D-glucose, D-xylose, L-arabinose and sucrose. To be selected, strains should grow on at least one of the C5 sugars.

*c* The final 35 selected strains were purified, resulting in 44 pure cultures, which were subsequently tested for genetic accessibility.
TABLE 2 Fermentation performance of transformable isolates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (h)</th>
<th>Lac</th>
<th>Ace</th>
<th>Suc</th>
<th>Mal</th>
<th>Eth</th>
<th>Total</th>
<th>Av. prod. (g/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. smithii</em> ET 138</td>
<td>±8</td>
<td>±3.03</td>
<td>±0.45</td>
<td>±0.00</td>
<td>nd</td>
<td>±0.22</td>
<td>±2.34</td>
<td>±0.01</td>
</tr>
<tr>
<td><em>G. thermodenitrificans</em> ET 144-2</td>
<td>±70</td>
<td>±3.26</td>
<td>±0.16</td>
<td>±0.22</td>
<td>±0.08</td>
<td>±0.30</td>
<td>±4.03</td>
<td>±0.01</td>
</tr>
<tr>
<td><em>G. thermodenitrificans</em> ET 251</td>
<td>±11</td>
<td>±1.38</td>
<td>±0.05</td>
<td>±0.04</td>
<td>nd</td>
<td>±0.06</td>
<td>±1.35</td>
<td>±0.01</td>
</tr>
</tbody>
</table>

The results shown are the averages of duplicates; standard deviations are indicated in italics after the “±”.


b Av. prod.: average productivity. Calculated by dividing total products by fermentation time.
TABLE 3 Fermentation of *B. smithii* ET 138 at different pHs.

<table>
<thead>
<tr>
<th>pH</th>
<th>Time (h)</th>
<th>Lac</th>
<th>Ace</th>
<th>Suc</th>
<th>Total</th>
<th>Av. prod. (g/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>43(^a)</td>
<td>3.3</td>
<td>0.3</td>
<td>0.0</td>
<td>3.6</td>
<td>0.08</td>
</tr>
<tr>
<td>5.5</td>
<td>43</td>
<td>9.9</td>
<td>0.3</td>
<td>0.1</td>
<td>10.3</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>141</td>
<td>14.0</td>
<td>0.6</td>
<td>0.1</td>
<td>14.7</td>
<td>0.10</td>
</tr>
<tr>
<td>6.5</td>
<td>43</td>
<td>10.5</td>
<td>0.3</td>
<td>0.0</td>
<td>10.8</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>141</td>
<td>21.1</td>
<td>0.6</td>
<td>0.1</td>
<td>21.8</td>
<td>0.15</td>
</tr>
</tbody>
</table>

\(^a\) At pH 4.5, no more acids were produced after 43 h so no further time points are shown.
\(^b\) Abbreviations: Lac: lactate, Ace: acetate, Suc: succinate.
\(^c\) Av. prod.: average productivity. Calculated by dividing total products by fermentation time.
TABLE 4 Optimization of electrotransformation for *B. smithii* ET 138.

<table>
<thead>
<tr>
<th>Parameter changed</th>
<th>Final OD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hrs of growth&lt;sup&gt;b&lt;/sup&gt;</th>
<th>kV</th>
<th>µF</th>
<th>Ω</th>
<th>Cuvette mm</th>
<th>µg DNA</th>
<th>Rec. medium&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CFU&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Settings&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.483</td>
<td>1.75</td>
<td>1.5</td>
<td>25</td>
<td>600</td>
<td>1</td>
<td>1</td>
<td>RG2</td>
<td>3</td>
</tr>
<tr>
<td>Settings&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.483</td>
<td>1.75</td>
<td>2.0</td>
<td>25</td>
<td>200</td>
<td>2</td>
<td>1</td>
<td>RG2</td>
<td>33</td>
</tr>
<tr>
<td>Settings</td>
<td>0.424</td>
<td>2.9</td>
<td>2.0</td>
<td>25</td>
<td>400</td>
<td>2</td>
<td>0.7</td>
<td>RG2</td>
<td>157</td>
</tr>
<tr>
<td>Rec. medium</td>
<td>0.519</td>
<td>1.75</td>
<td>2.0</td>
<td>25</td>
<td>400</td>
<td>2</td>
<td>1</td>
<td>LB2</td>
<td>960</td>
</tr>
<tr>
<td>Fast growth&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.539</td>
<td>1</td>
<td>2.0</td>
<td>25</td>
<td>400</td>
<td>2</td>
<td>2.5</td>
<td>LB2</td>
<td>1900</td>
</tr>
<tr>
<td>µg DNA added&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.617</td>
<td>1.3</td>
<td>2.0</td>
<td>25</td>
<td>400</td>
<td>2</td>
<td>0.2</td>
<td>LB2</td>
<td>2409</td>
</tr>
<tr>
<td>µg DNA added&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.617</td>
<td>1.3</td>
<td>2.0</td>
<td>25</td>
<td>400</td>
<td>2</td>
<td>0.02</td>
<td>LB2</td>
<td>5118</td>
</tr>
</tbody>
</table>

<sup>a</sup> Final OD<sub>600</sub> after the indicated number of hours (<sup>b</sup>) when growing cells prior to making them competent.

<sup>b</sup> Number of hours cells had grown before making them competent.

<sup>c</sup> RG2 is LB2 with 121 g/L sucrose and 10 g/L glucose (29).

<sup>d</sup> CFU: colony forming units per µg DNA. Using fresh cells or cells stored at -80°C did not change CFUs.

<sup>e</sup> Settings from (29).

<sup>f</sup> Settings based on (30), same settings as used for screening of *Geobacillus* strains.

<sup>g</sup> In these experiments, after overnight growth, cells were transferred to 500 mL Erlenmeyer flasks or 1 L bottles (having a similar bottom surface) instead of to a 250 mL Erlenmeyer to allow for more aeration.