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Note

An improved agar medium for growth of *Geobacillus thermoglucosidarius* strains

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ABSTRACT

Geobacillus species have potential applications in many biotechnological processes. They are fastidious in their vitamin and amino acid requirements. A new semi-defined agar medium (SDM) was developed which gave consistently high viable cell counts of various *G. thermoglucosidasius* strains (5×10^8–6×10^8 cfu/ml) under aerobic conditions at 70 °C.

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1. Introduction

The genus *Geobacillus* includes thermophilic Gram-positive spore-forming bacteria that form a phylogenetically coherent clade within the family *Bacillaceae*. These thermophiles seem to be ubiquitous and viable *Geobacillus* spores can be isolated in large quantities from hot environments such as hydrothermal vents. Paradoxically, they are also present in cool soils and cold ocean sediments (Zeigler, 2014).

The *Geobacillus* spp. are thermophilic aerobic or facultative anaerobic bacilli (Nazina et al., 2001). Members of the genus are capable of growth between 40 and 70 °C and can ferment both hexose and pentose sugars and oligomers to generate lactate, formate, acetate and ethanol as products (Baker et al., 1953; Amartey et al., 1991). They are of interest for biotechnology as sources of thermostable enzymes and natural products. Industrially important enzymes originating from *Geobacillus* spp. include lipases (Schmidt-Dannert et al. (1998), glycoside hydrolases (Fridjonsson et al., 1999; Bartosiak-Jentys et al., 2013; Suzuki et al., 2013), *N*-acylhomoserine lactonase (Seo et al., 2011) and DNA polymerase I (Sandalli et al., 2009) and protease (Chen et al., 2004) among others. *Geobacillus* spp. are also used as cellular factories for heterologous expression of enzymes and as hosts for directed evolution (Wiegel et al., 1985; Niehaus et al., 1999; Couñago and Shamoo, 2005; Marchant et al., 2006; Taylor et al., 2009; Tabachnikov and Shoham, 2013).

Furthermore, many innovative environmentally friendly processes have also been developed using many other biocatalysts from thermophiles. These include degradation of hazardous aromatic compounds from oil refineries, petrochemical plants, coal conversion plants and phenol resin industries (McMullan et al., 2004; Payton and Hartley, 1985; Baker et al., 1953). Less positively, *Geobacillus* spp. are common contaminants in the dairy and food industries (Burgess et al., 2010).

Our previous and on-going studies with *Geobacillus thermoglucosidasius* (NCIMB 11955) the parent strain have demonstrated its potentials and those of various isolated mutants strains (LLD-R, LLD-15, LLD-16, T13-N, FAC-N2) for improved fermentative production of ethanol from sugars derived from lignocellulose. *Geobacillus thermoglucosidasius* (NCIMB 11955) has an optimum growth temperature and pH of 65 °C and 7.0 respectively. It is a mixed acid producer that ferments many carbohydrates to mainly lactate with formate, acetate and ethanol. Accumulation of the organic acids in the growth medium causes a rapid fall in pH that inhibits further growth of the cell. A pH < 6.2 has been found to completely inhibit growth of the cells (Payton and Hartley, 1985; Hartley and Shama, 1987; Amartey et al., 1991; San Martin et al., 1994; Javed, 1993; Taylor, 2007).

Like most thermophiles, *Geobacillus* species are extremely fastidious in their vitamin and amino acid requirements and their complex nutritional requirements are usually satisfied with high levels of undefined compounds such as tryptone, peptones, meat and yeast extract or similar complex nutritional sources (Lee et al., 1982; Cripps et al., 2009).

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However, for metabolic engineering, mutagenesis, physiological and many fermentation studies of these strains, defined/minimal media (liquid and agar) that support good growth and gives consistently high viable cell counts are often required to elucidate the effects of changes in medium composition on cell growth and product formation, calculation of mass balances and for isolating auxotrophic mutants.

Although a defined liquid media for aerobic and anaerobic growth/fermentation of various carbohydrates by these stains have been developed (Baker et al., 1953; Cripps et al., 2009; San Martin et al., 1992), the absence of a defined/minimal agar medium for growth of these strains means they are routinely grown on agar media containing complex compounds such as Lauria broth (LB) agar, nutrient agar and 2TY (tryptone-yeast extract) agar. We have found that growth of these strains on these rich media is most often inconsistent and non-reproducible**.**

There is therefore the need for an agar medium (defined/minimal) that will support good growth with high and reproducible viable cell counts of *Geobacillus thermoglucosidasius* strains and other *Geobacillus* species.

In our quest for a minimal agar medium for growing these strains, we have developed a semi-defined agar medium (SDM) that contains a dual carbon source (glycerol and pyruvate) and very low levels of yeast extract 0.002 g/l and tryptone 0.002 g/l and inorganic salts The main components of the semi-defined agar medium (SDM) are (g/l): tryptone 0.2; yeast extract 0.2; citric acid 0.32; di-sodium hydrogen orthophosphate (anhydrous) 2.0; magnesium sulphate (hepta-hydrate) 0.4; potassium sulphate 0.3; ammonium chloride 2.0; manganese chloride (tetra-hydrate) 0.003; ferric chloride 0.007; agar 15 and 1 ml of trace elements solution (mg/l); $ZnSO_4 \cdot \cdot 7H_2O$ 400; H_3BO_3 10; $CoCl_2 \cdot 6H_2O$ 50; $CuSO_4$ 200; $NiCl_2 \cdot 6H_2O$ 10 and EDTA 250). Glycerol 5 g/l and pyruvate 5 g/l are the main carbon sources. Preparation of the SDM involves the glycerol and pyruvate being prepared together but separate from the other constituents and mixed after sterilisation at 121 °C. Final pH of the medium should be made to 7 with 1 M NaOH.

Growth of various *G. thermoglucosidasius* mutant strains (LLD-R, LLD-15, LLD-16, FAC-T13, FAC-N9, and FAC-N22) on the SDM containing both pyruvate and glycerol was compared with those containing pyruvate and glycerol separately, glucose and other agar media in terms of viable cell counts after 24 h of growth. The strains were first grown aerobically in 100 ml of YT medium (pH 7.0) (g/l): Yeast extract 25; tryptone 12.5 at 70 °C, 200 rpm to an optical density ($OD_{600\ nm}$) of 1.0. The cells were then harvested at 15,000 rpm for 15 min and washed twice with physiological saline solution (pH 7). Serial dilutions (10^{-1} to 10^{-7}) of the cell suspension were made in a physiological saline solution and 100 μl of cell suspension of each dilution were plated out in triplicate on the various agar media that have been pre-warmed at 60 °C for about 20 min. Colonies were counted after incubation for 24 h at 70 °C. All the tests were done in triplicate.

Results presented in Table 1 show that the average viable cell count of *G. thermoglucosidasius* LLD-16 was higher and much more reproducible on the medium containing the combination of glycerol and pyruvate than those containing the individual carbon substrates, the Yeast extract-tryptone agar medium (YT) and also the YT medium containing a combination of glycerol and pyruvate. Table 2 shows that all the *G. thermoglucosidasius* strains grew well and gave reproducible and comparable viable cell counts on the SDM with glycerol and pyruvate as carbon sources.

The reason why the SDM containing pyruvate and glycerol supported better growth of the strains compared to the other carbon sources is currently being investigated. Further work is also currently being done to remove the tryptone and the yeast extract completely from this medium and make it a minimum medium.

We routinely use this medium for genetic and metabolic studies of these strains and also for short-term maintenance of the strains.

Table 1
Average viable cell counts of *G. thermoglucosidasius* LLD-16 on the different agar media after overnight aerobic growth at 70 °C.

Agar media	No. of colonies/ml of culture ($OD_{600} = 1.0$)[a]
SDM + D-glucose	1×10^8 (0.5)
SDM + pyruvate	2×10^8 (0.1)
SD M + glycerol	3×10^8 (0.1)
SDM + glycerol and pyruvate	6×10^8 (0.04)
Yeast extract-tryptone (YT)	3×10^7 (0.80)
Yeast extract-tryptone (YT) + glycerol and pyruvate	3×10^8 (0.5)

[a] Average values obtained for the 3 replicates. Values in brackets are standard deviations between the replicates.

Table 2
Comparison of viable cell counts of the various *G. thermoglucosidasius* strains on SDM (glycerol and pyruvate) after overnight aerobic growth at 70 °C.

G. thermoglucosidasius strain	No. of colonies/ml of culture ($OD_{600} = 1.0$)[a]
LLD-R	5×10^8
LLD-15	5×10^8
LLD-16	6×10^8
T13-N	5×10^8
FAC-N22	6×10^8

[a] Average values for the 3 replicates.

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References

Amartey, S.A., Leak, D.J., Hartley, B.S., 1991. Development and optimisation of a defined medium for aerobic growth of *Bacillus stearothermophilus* LLD-15. Biotechnol. Lett. 13 (9), 621–626.
Baker, H., Sobotka, H., Hunter, S.H., 1953. Growth requirements of some thermophilic and mesophilic *Bacilli*. J. Gen. Microbiol. 9, 485–493.
Bartosiak-Jentys, J., Hussein, A.H., Lewis, C.J., Leak, D.J., 2013. Modular system for assessment of glycosyl hydrolase secretion in *Geobacillus thermoglucosidasius*. Microbiology 159, 1267–1275.
Burgess, S.A., Lindsay, D., Flint, S.H., 2010. *Thermophilic bacilli* and their importance in dairy processing. Int. J. Food Microbiol. 144, 215–225.
Chen, X.-G., Stabnikova, O., Tay, J.-H., Wang, J.-Y., Tay, S.T.-L., 2004. Thermoactive extracellular proteases of *Geobacillus caldoproteolyticus*, sp. nov., from sewage sludge. Extremophiles 8, 489–498.
Couñago, R., Shamoo, Y., 2005. Gene replacement of adenylate kinase in the gram-positive thermophile *Geobacillus stearothermophilus* disrupts adenine nucleotide homeostasis and reduces cell viability. Extremophiles 9, 135–144.
Cripps, R.E., Eley, K., Leak, D.J., Rudd, B., Taylor, M., Todd, M., Boakes, S., Maerin, S., Atkinson, T., 2009. Metabolic engineering of *Geobacillus thermoglucosidasius* for high ethanol production. Metab. Eng. 11, 398–408.
Fridjonsson, O., Watzlawick, H., Gehweiler, A., Mattes, R., 1999. Thermostable alpha-galactosidase from *Bacillus stearothermophilus* NUB3621: cloning, sequencing and characterization. *FEMS* Microbiol. Lett. 176, 147–153.
Hartley, B.S., Shama, G., 1987. Novel ethanol fermentations from sugar cane and straw. Phil. Trans. Roy. Soc. Lond. A 321, 555–568.
Javed, M., 1993. Strain improvement of a thermophilic *Bacillus* for ethanol production. Ph.D thesis. Imperial College London.
Lee, Y.H., Brown, M.R.W., Cheung, H.Y., 1982. Defined minimal media for the growth of prototrophic and auxotrophic strains of *Bacillus stearothermophilus*. J. Appl. Bacteriol. 53, 179–187.
Marchant, R., Sharkey, F.H., Banat, I.M., Rahman, T.J., Perfumo, A., 2006. The degradation of *n*-hexadecane in soil by thermophilic geobacilli. FEMS Microbiol. Ecol. 56, 44–54.
McMullan, J.M., Christie, T.M., Rahman, I.M., Banat, I.M., Ternan, N.G., Marchant, R., 2004. Habitat, applications and genomics of the aerobic, thermophilic genus *Geobacillus*. Biochem. Soc. Trans. 32 (2), 214–217.
Nazina, T.N., Tourova, T.P., Poltaraus, A.B., Novikova, E.V., Grigoryan, A.A., Ivanova, A.E., Lysenko, A.M., Petrunyaka, V.V., Osipov, G.A., Belyaev, S.S., Ivanov, M.V., 2001. Taxonomic study of aerobic thermophilic bacilli. Int. J. Syst. Evol. Microbiol. 51, 433–446.
Niehaus, F., Bertoldo, C., Kähler, M., Antranikian, G., 1999. Extremophiles as a source of novel enzymes for industrial application. Appl. Microbiol. Biotechnol. 51, 711–729.
Payton, M.A., Hartley, B.S., 1985. Mutants of *Bacillus stearothermophilus* lacking NAD-linked L-lactate dehydrogenase. FEMS Microbiol. Lett. 26, 333–336.

San Martin, R., Bushell, D., Leak, D.J., Hartley, B.S., 1992. Development of a synthetic medium for continuous anaerobic growth and ethanol production with a lactate dehydrogenase mutant of *Bacillus stearothermophilus*. J. Gen. Microbiol. 138, 987–996.

San Martin, R., Bushell, D., Leak, D.J., Hartley, B.S., 1994. Cultivation of an L-lactate dehydrogenase mutant of *Bacillus stearothermophilus* in continuous culture with cell recycle. Biotechnol. Bioeng. 44, 21–28.

Sandalli, C., Singh, K., Modak, M.J., Ketkar, A., Canakci, S., Demir, I., Belduz, A.O., 2009. A new DNA polymerase I from *Geobacillus caldoxylosilyticus* TK4: cloning, characterization, and mutational analysis of two aromatic residues. Appl. Microbiol. Biotechnol. 84, 105–117.

Schmidt-Dannert, C., Pleiss, J., Schmid, R.D., 1998. A toolbox of recombinant lipases for industrial applications. Ann. N. Y. Acad. Sci. 864, 14–22.

Seo, M.-J., Lee, B.-S., Pyun, Y.-R., Park, H., 2011. Isolation and characterization of *N*-acylhomoserine lactonase from the thermophilic bacterium, *Geobacillus caldoxylosilyticus* YS-8. Biosci. Biotechnol. Biochem. 75, 1789–1795.

Suzuki, H., Okazaki, F., Kondo, A., Yoshida, K., 2013. Genome mining and motif modifications of glycoside hydrolase family 1 members encoded by *Geobacillus kaustophilus* HTA426 provide thermostable 6-phospho-βglycosidase and β-fucosidase. Appl. Microbiol. Biotechnol. 97, 2929–2938.

Tabachnikov, O., Shoham, Y., 2013. Functional characterization of the galactan utilization system of *Geobacillus stearothermophilus*. FEBS J. 280, 950–964.

Taylor, M.P., 2007. Metabolic Engineering of *Geobacillus* Species for Enhanced Ethanol Production. Ph.D thesis. Imperial College London.

Taylor, M.P., Eley, K.L., Martin, S., Tuffin, M.I., Burton, S.G., Cowan, D.A., 2009. Thermophilic ethanologenesis: future prospects for second-generation bio-ethanol production. Trends Biotechnol. 27, 398–405.

Wiegel, J., Ljungdahl, L.G., Demain, A.L., 1985. The importance of thermophilic bacteria in biotechnology. Crit. Rev. Biotechnol. 3, 39–108.

Zeigler, D.R., 2014. The *Geobacillus* paradox: why is a thermophilic bacterial genus so prevalent on a mesophilic planet? Microbiology 160, 1–11.