Exploring the potential of *Bacillus subtilis* as cell factory for food ingredients and special chemicals

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Key words: *Bacillus subtilis*; Fermentation; Primary metabolites

Abstract

Background

Bacillus subtilis has been established as model microorganism for fundamental research in the laboratory on protein production/secretion and sporulation and as model bacterium for controlling spoilage in the food industry. It has also been used for production of (commercial) enzymes and several secondary metabolites such as vitamins. However, this doesn't fully reflect the potential of *B. subtilis* as a cell-factory. Here, various strains of *B. subtilis*, including food-grade, spore-deficient strains and industrially used strains, were compared for their growth and metabolic potential. Industry-relevant parameters were analyzed for all strains under various aeration regimes, under anaerobic conditions, in various nutritious and nutrient-limited cultivation media, with and without organic nitrogen sources, and with and without sugar.

Results

Practical experiments were conducted to compare industrial relevant properties like growth rates, intracellular components and extracellular metabolite profile of different *B. subtilis* strains. Based on growth flexibility in different media, we found that some strains like NCIB3610 and DSM1092 are adapted to inorganic or organic nitrogen source utilization,

which is highly relevant when considering a biorefinery approach using various cheap and abundant waste/sidestreams. Secondly, spore-deficient strains such as 3NA, 168S and PY79S, showed advantages in microbial protein and acetolactate pathway expression, which is associated with applications in food industry for protein supplement and diacetyl production. Lastly, WB800 and PY79S exhibited potential for fermentative production of Dipicolinic acid, 2,3-Butanediol and Lactic acid that could serve as precursors for biopolymers.

Conclusion

This study demonstrates the broad potential for more extensive industrial use of *Bacillus subtilis* in the (bio-based) chemical industry for use of sidestreams, in the personal care industry, in the food industry for food additive production, and in the bio-sustainable industry for biofuel and bio-degradable plastic precursors production. In addition, selecting different *B. subtilis* strains for specific purposes makes full use of the diversity of this species and increases the potential of *B. subtilis* in its contribution to the bio-based economy.

Introduction

As the market for sustainable chemical production expands, researchers have been exploring bioproduction of chemicals for several decades. This includes the use of microbial fermentation to produce chemicals of interest. Compared with conventional chemical approach, microbial cell factories require lower temperature and pressure, and can convert renewable substrates into products of interest. Some model microorganisms like *Escherichia coli*[1], *Bacillus subtilis*[2] and *Saccharomyces cerevisiae*[3] are promising candidates for industrial-relevant chemicals production due to their developed databases and extensive molecular toolbox.

B. subtilis is a representative gram-positive bacterium that has been intensively used in studies of sporulation process and protein production/excretion because of its robustness as a spore former and its excellent protein secretory capability[4]. In addition, it is recognized as

51 a plant growth-promoting bacterium (PGPB), that enhances plant growth and protects plants
52 from phytopathogen[5], instead of chemical fertilizers and pesticides, can contribute to an
53 eco-friendly and sustainable economy. Moreover, different *B. subtilis* strains have been
54 developed through mutagenesis or metabolic engineering to metabolize various
55 carbon/nitrogen sources into several secondary metabolites such as (commercial) enzymes[6]
56 and vitamins[7] or into biochemicals, such as oligosaccharides[8] and organic acids[9].
57 Among all *B. subtilis* strains, *B. subtilis* 168[10] is the major workhorse for versatile cell factory
58 construction because of its sufficient gene annotations and high transformation efficiency.
59 However, focusing solely on *B. subtilis* 168 could ignore the full potential of this species as
60 many variants have unique advantages in environment adaption or metabolism (Fig. 1). For
61 example, *B. subtilis* 3NA[11] can reach high cell density during fed-batch fermentation, which
62 is an attractive point, but only limited work has been done to explore its potential. Also, *B.*
63 *subtilis* PY79[12] owns a large amount of -omics data that can help metabolic rewiring, but it
64 is mainly used in studies related to spores and biofilm[13]. Therefore, we propose that current
65 research don't fully reflect the potential of *B. subtilis* as a cell-factory, and systematic
66 evaluation of metabolic potential of different *B. subtilis* strains may help expand the
67 application of this species in more aspects in the bio-based industry (The difference and
68 details of all selected *B. subtilis* strains are discussed in the "Strains and media" section).
69 In this research, we investigated metabolic potential of nine *B. subtilis* variants in view of
70 extending the use of this model species. First, we assessed the metabolic flexibility of *B.*
71 *subtilis* strains regarding various media. Second, the effect of dissolved oxygen on growth
72 and extracellular metabolites accumulation were investigated in flask level. Finally, we
73 discussed the possibilities of different *B. subtilis* strains acting as cell factories and make
74 practical recommendations for strain selection for industrial applications for different
75 purposes.

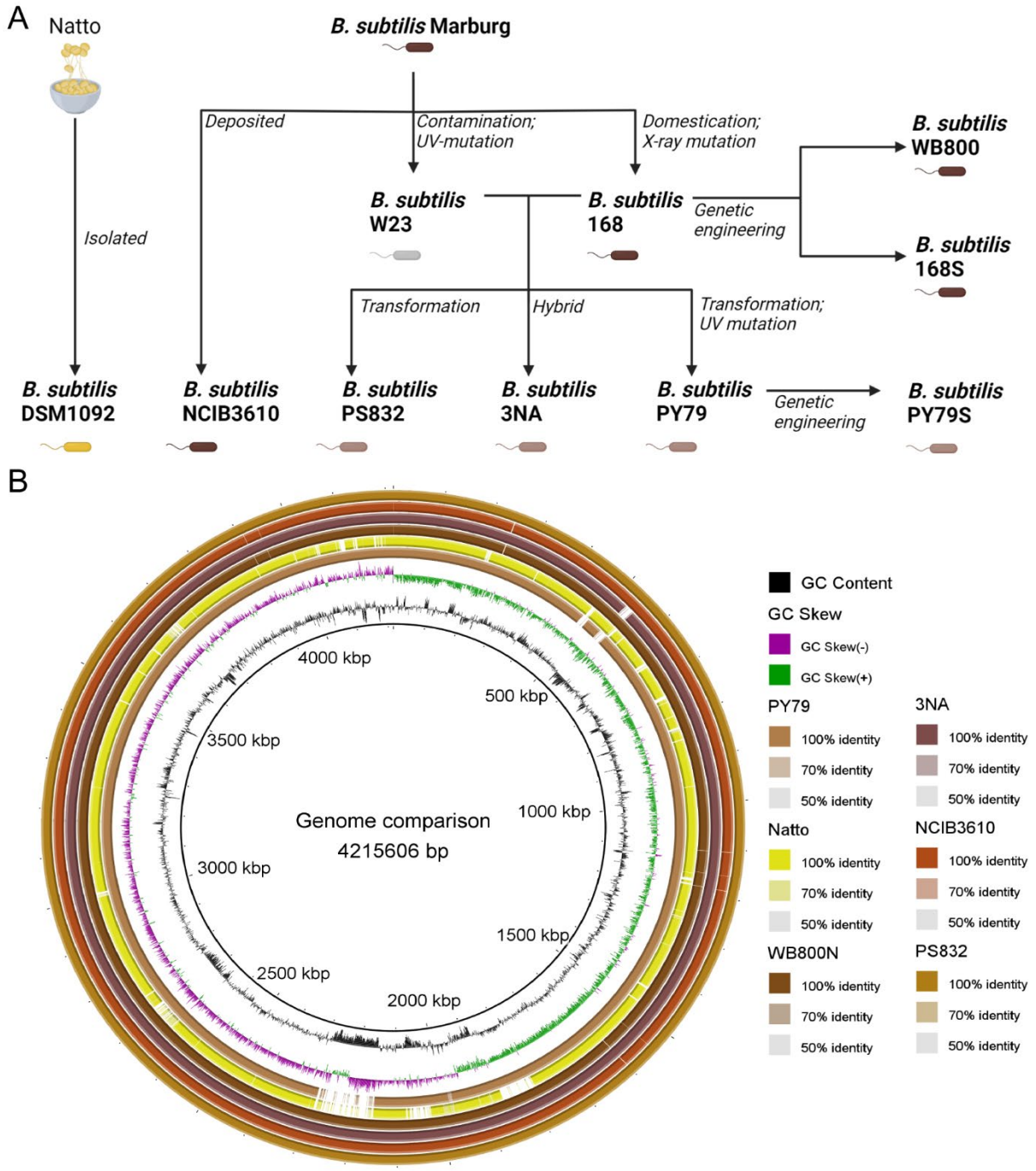


Figure 1 | A. Genome heritage of nine *B. subtilis* strains. B. Genomic sequence comparison of representative *B. subtilis* strains using genome of 168 as the reference.

Results

Variation in growth characteristics of *B. subtilis* strains associated with medium

To investigate the metabolic flexibility on different nitrogen sources of *B. subtilis* strains, we cultivated nine *B. subtilis* strains on different media in 96-well plates and plotted their specific

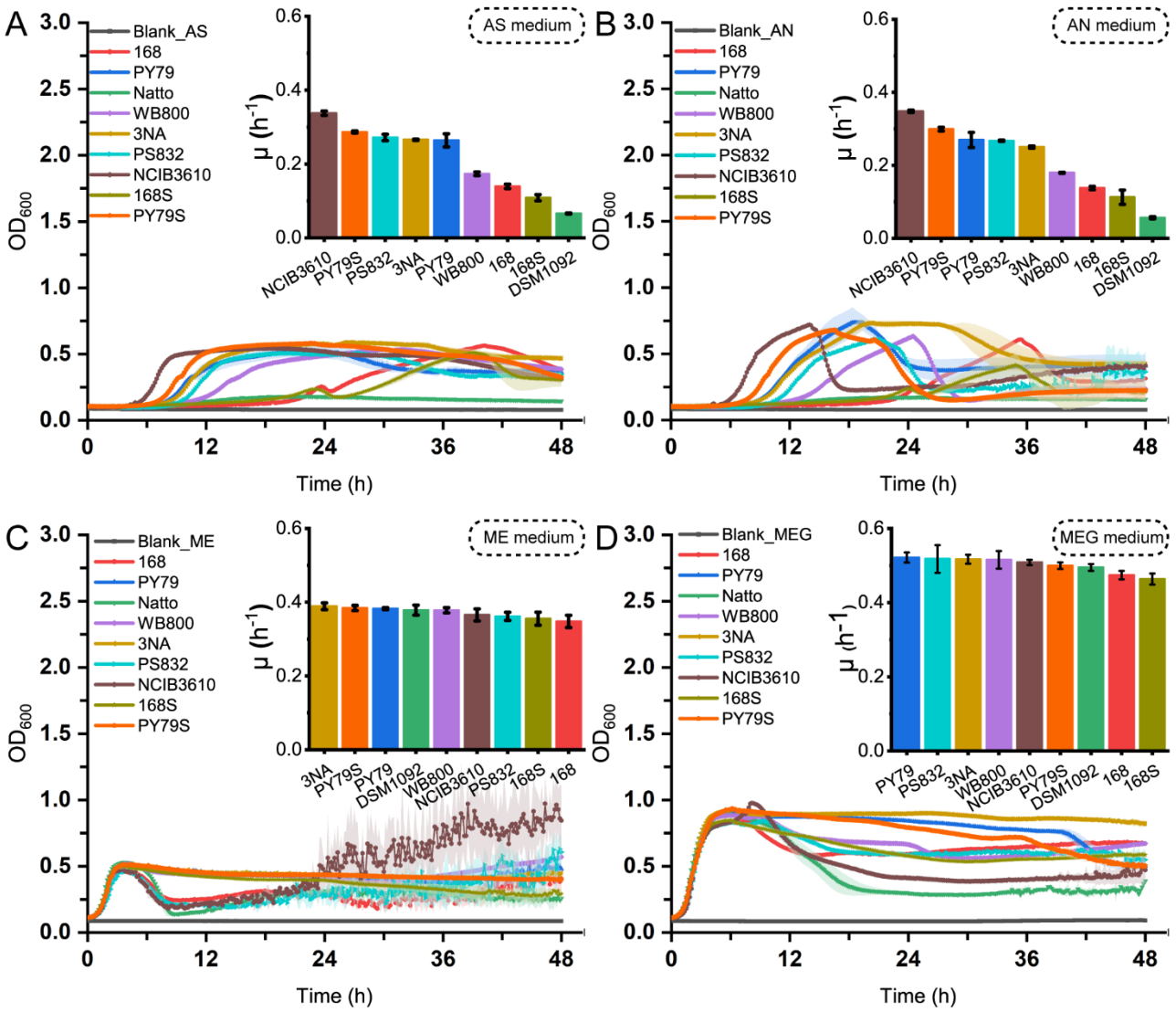
growth rates (μ) and growth curves in Fig. 2. The specific growth rates of the strains were pairwise compared by one-way ANOVA (Supplementary Fig 1).

B. subtilis NCIB3610[14], PY79S, PS832[15], 3NA and PY79 exhibited higher specific growth rates than other strains in media with ammonium salts (Fig. 2A, Fig. 2B), suggesting that they are competitive in these environments. PS832, 3NA and PY79 showed no significant difference in specific growth rate in the media containing ammonium salts. *B. subtilis* NCIB3610, however, showed significant difference with other strains with the highest growth rate of 0.33 h^{-1} and 0.35 h^{-1} among all strains in media with ammonium sulfate and with ammonium nitrate, respectively (Fig. 2A, Fig. 2B). Subsequently, we replaced the ammonium salts and glucose with meat extract (ME medium) to assess the growth characteristics of *B. subtilis* strains in the environment containing free amino acids, peptides and proteins. In ME medium, most strains showed no significant difference in specific growth rates with others. Notably, the growth rate of DSM1092[16] increased from 0.06 h^{-1} to 0.38 h^{-1} after switching the nitrogen source from inorganic to organic, suggesting that it is adapted for the environment containing organic nitrogen source (Fig. 2C). By further adding glucose into ME medium (MEG medium), we observed that the growth rate of DSM1092 increased by 28.9% from 0.38 h^{-1} to 0.49 h^{-1} (Fig. 2D).

In addition to growth rates, *B. subtilis* strains exhibited different growth patterns when growing in different media. In the media containing organic nitrogen sources, most strains showed immediate propagation and started to decline after reaching the peak, whereas the downward trend was not the same for different strains (Fig. 2C, Fig. 2D). In media with ammonium salts, all strains presented obvious lag phases (Supplementary table 1) and long stationary phase when growing in the medium with ammonium sulfate (AS medium) (Fig. 2A). Unlike the abovementioned cases, strains showed both different lag phases and a decreasing trend

107 after reaching the highest biomass in the medium with ammonium nitrate (AN medium) (Fig.

108 2B).



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110 **Figure 2** | Growth curves and specific growth rates of nine *B. subtilis* strains on various media. A. Growth

111 properties of nine strains in medium with ammonium sulphate; B**.** Growth properties of nine strains in medium

112 with ammonium nitrate; C**.** Growth properties of nine strains in medium with meat extract; D. Growth properties

113 of nine strains in medium with meat extract and glucose. The shallow surrounding growth curve represents the

114 standard deviations of replicates.

115 Variation in growth pattern of *B. subtilis* strains associated with oxygen

116 To evaluate the effect of oxygen on *B. subtilis* growth, we created high Dissolved oxygen

117 (DO), optimal DO and oxygen-deprived conditions for fermentation experiments in flask and

118 measured OD_{600} values of strains in 72h. When ammonium salt was used as the only nitrogen
119 source, only the growth of NCIB3610 showed an obvious improvement with increase of
120 dissolved oxygen(Fig. 3A, Fig. 3B). However, in the medium containing free amino acids, all
121 strains grew faster with the increase of dissolved oxygen. For example, in the medium with
122 meat extract and glucose (MEG medium), the OD_{600} value of *B. subtilis* DSM1092 reached
123 16, which was 3 times the value under optimal DO condition (Fig. 3D). Furthermore, as the
124 protein and polypeptide content in the medium decreased, the influence of dissolved oxygen
125 on the growth pattern of the strain became less. When casein hydrolysate was the only
126 organic nutrient in the medium, elevating dissolved oxygen had no effect on the growth of all
127 strains (Supplementary Fig. 2C).
128 Additionally, we observed that *B. subtilis* strains only grew in the presence of both glucose
129 and nitrate or both glucose and organic nitrogen sources under oxygen-deprived condition.
130 However, NCIB3610 reached 0.5 in the medium with ammonium nitrate (AN medium), far
131 exceeding other variants (Fig. 3B). Moreover, 3NA and PY79 whose highest OD_{600} were both
132 1.4, outpaced other strains by faster growth and higher biomass in MEG medium (Fig. 3D).

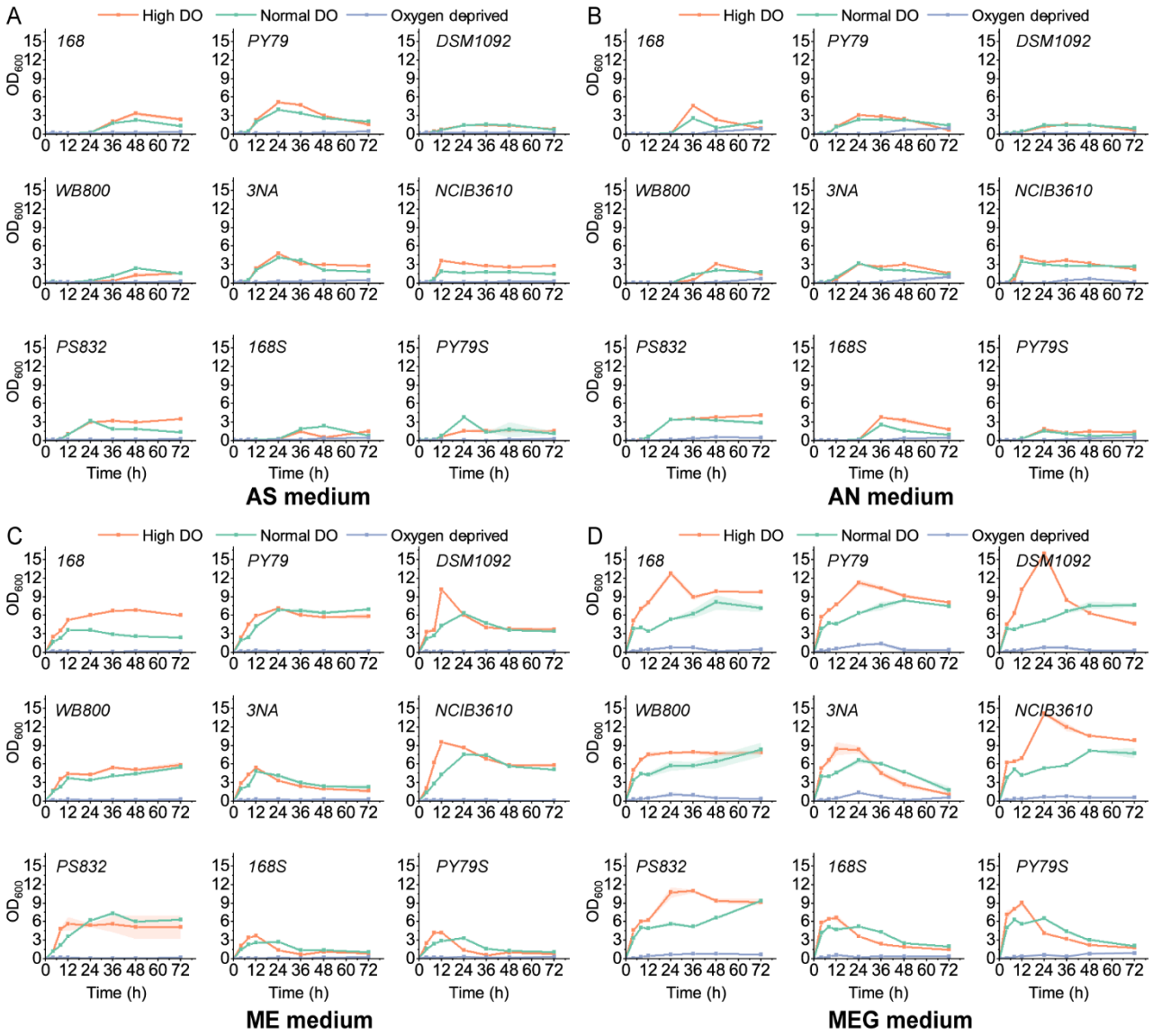

Figure 3 | A~D. Growth curves of strains in flasks in medium with ammonium sulphate (AS), ammonium nitrate (AN), meat extract (ME) and both meat extract and glucose (MEG) under high DO, optimal DO and oxygen-deprived conditions. The shallow surrounding growth curve represents the standard deviations of replicates.

Variation in intracellular components of *B. subtilis* strains

After comparing growth flexibility of strains under different conditions, we turned to cellular components of different strains, such as microbial protein and spores. To compare the protein in biomass and dry cell weight of *B. subtilis* strains, we conducted fermentation experiments in LB medium supplied with glucose (LBG medium) in 250-ml flasks for 24h. At 6h (exponential phase), *B. subtilis* strains showed similar dry cell weight and protein proportions,

143 while the protein ratios of strains varied from 11% to 38% at 24h (stationary phase) (Table
144 1). At 24h, spore-deficient *B. subtilis* 3NA exhibited the highest proportion of 38%, 3.4 times
145 higher than that of PY79 which is a well-known spore-forming variant. The protein proportion
146 of PY79 also increased from 11% to 36% after we disrupted its sporulation via knocking out
147 *spo0A* (PY79S), indicating the negative effect of sporulation on microbial protein proportion.

Table 1 Dry cell weight and protein concentration of strains per OD_{600}

Strains	6h			24h		
	Dry weight (mg/l)	**Protein concentration (mg/l)**	**Proportion Protein/Dry weight (%)**	**Dry weight (mg/l)**	**Protein concentration (mg/l)**	**Proportion Protein/Dry weight (%)**
MG1655	530 ± 23	89 ± 9	17 ± 1	288 ± 4	113 ± 15	39 ± 6
168	415 ± 30	48 ± 14	11 ± 3	425 ± 42	77 ± 10	18 ± 1
PY79	390 ± 41	57 ± 9	14 ± 2	500 ± 16	53 ± 6	11 ± 1
DSM1092	355 ± 122	68 ± 12	21 ± 7	538 ± 31	82 ± 5	15 ± 0
WB800	400 ± 67	58 ± 17	21 ± 10	400 ± 8	123 ± 24	31 ± 8
3NA	415 ± 33	68 ± 5	16 ± 0	325 ± 47	122 ± 16	38 ± 12
NCIB3610	440 ± 12	66 ± 8	15 ± 2	260 ± 69	66 ± 9	27 ± 6
PS832	435 ± 18	73 ± 5	17 ± 1	210 ± 56	38 ± 5	19 ± 3
168S	460 ± 34	72 ± 9	16 ± 1	588 ± 37	166 ± 2	28 ± 2
PY79S	420 ± 32	51 ± 12	12 ± 3	475 ± 15	171 ± 2	36 ± 1

148 Although *B. subtilis* spores are usually discussed with food spoilage and contamination, they
149 are also related to dipicolinic acid (DPA) production which is industrial relevant. To determine
150 spore dry weight and DPA content in the spores of *B. subtilis* strains, we cultivated strains in
151 MOPS medium in 250-ml flasks for 72h, and purified spores. As a result, we didn't find DPA
152 in supernatant of cultures but only in spores. *B. subtilis* 168 stored 74 mg of DPA in 1 g of
153 dry spore, which is the lowest among selected strains (Table 2). However, two mutants
154 WB800[17] and PS832 derived from 168 showed the highest DPA production, namely 128
155 mg and 126 mg of DPA in 1 g of dry spore, respectively.

Table 2 Spore properties of strains

Strains	Dry weight (g/l/OD)	Dipicolinic acid concentration (mg/l/OD)	Proportion (mg_{DPA}/g_{spore})
168	0.09 ± 0.00	6.68 ± 0.19	74.56 ± 0.87
PY79	0.11 ± 0.01	10.22 ± 0.16	91.13 ± 8.25
DSM1092	0.13 ± 0.00	7.56 ± 0.15	56.36 ± 2.43
WB800	0.10 ± 0.01	12.29 ± 0.21	127.59 ± 10.32
NCIB3610	0.10 ± 0.00	8.77 ± 0.12	90.77 ± 0.66
PS832	0.05 ± 0.00	6.55 ± 0.20	126.34 ± 12.88

156 ## Variation in extracellular metabolites distribution of *B. subtilis* strains

157 To further investigate the metabolic distribution during fermentation, the profiles of organic
158 acids and some primary metabolites were determined. Firstly, we observed pathway
159 preference of *B. subtilis* strains over time. For example, under optimal Dissolved Oxygen (DO)
160 condition, the acetolactate pathway had a greater flux than other two pathways at 12h in
161 DSM1092 in MEG medium while the acetate synthesis pathway took over at 24h (Fig. 4A).
162 Under high DO condition, however, lactate production outcompeted other two directions at
163 12h and we didn't see intermediate in the acetolactate pathway at 24h.

164 We take acetoin as an indicator for the acetolactate pathway because acetoin is the main
165 intermediate in this pathway, which also leads to Diacetyl and 2,3-Butanediol production.
166 Under aerobic conditions (Fig. 4B), acetoin was mainly produced in the first 24h and was
167 converted later (to butanediol) in ME and MEG medium but slowly accumulated in AS medium
168 under aerobic conditions. An interesting finding is that *B. subtilis* strains produced the lowest
169 titers of acetoin in ME medium in which glucose was absent, suggesting an important role for
170 glucose in the acetolactate pathway. 168S and PY79S producing the most acetoin both at 29
171 mM in MEG medium while 168, PY79, DSM1092, 3NA and PS832 depleted all acetoin after
172 48h in MEG medium (Fig. 4B).

173 Then we assessed the acetate synthesis pathway. The most interesting finding was two
174 different patterns of acetate production between *B. subtilis* DSM1092 and PS832 in MEG

175 medium under aerobic conditions. Acetate concentrations in the supernatants of PS832
176 fermentation were 0, 24.1 and 49.7 mM in 3 days, respectively (Fig. 4B). In contrast, the
177 corresponding data in the supernatant of DSM1092 fermentation were 0, 48.4 and 4.1 mM,
178 respectively. The continuous accumulation of acetate by PS832 indicated the risk of wasting
179 preferred carbon sources to by-products during fermentation while DSM1092 showed the
180 potential for growth on overflow metabolites.
181 In addition to the above two pathways that were strongly expressed under aerobic conditions,
182 we also observed that under oxygen-deprived condition, lactate is the main product of most
183 strains due to NAD^+ regeneration during lactate synthesis. In all conditions, *B. subtilis* PY79S
184 produced 42.36 mM lactate in MEG medium which was the highest among variants (Fig. 4B).

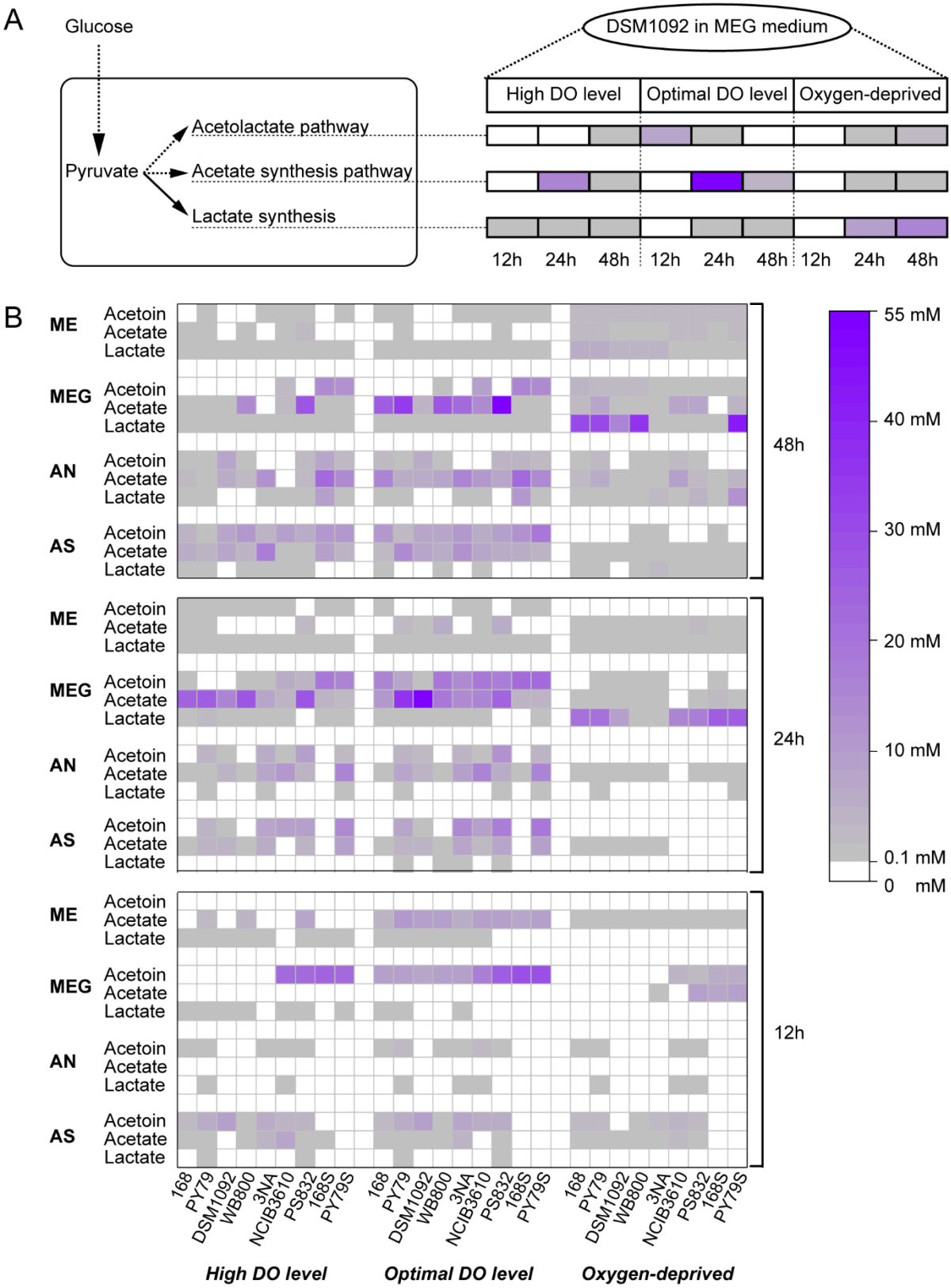

Figure 4 | A. Metabolite profile of DSM1092 in MEG medium. B: Metabolic distribution of nine *B. subtilis* strains in medium with ammonium sulphate (AS), ammonium nitrate (AN), meat extract (ME) and both meat extract and glucose (MEG) under high DO, optimal DO and oxygen-deprived conditions.

189 ## Discussion

190 In this study, we investigated metabolic flexibility of different *B. subtilis* strains corresponding
191 to various industrial applications and recommended certain variants for specific usage (Table
192 3).

Table 3 Proposed industrial applications based on experiments

Experiment results	Industrial application	Examples	Recommended strains
Growth properties	Biorefinery	Inorganic nitrogen	NCIB3610, PY79
		Protein rich	DSM1092, NCIB3610
		Oxic	DSM1092, NCIB3610
		Anoxic	PY79, 3NA
Biofilm	Personal care	Probiotic protection	NCIB3610, PS832
Biomass	Food additives	Microbial protein	Spore-deficient strains
Spores	Biopolymer precursor	Dipicolinic acid	WB800, PS832
Metabolites	Food additives	Diacetyl	168S, PY79S
	Biofuel, Biopolymer precursor	2,3-Butanediol	168S, PY79S
	Biopolymer precursor	Lactate	PY79S, 3NA

193 ### Biorefinery

194 For cost-effective production of bio-based chemicals and (food) ingredients, it is essential
195 that cell-factories can be productive based on cheap and abundantly available biomass,
196 waste- and sidestreams. Using such feedstocks, the preferred cell-factories will need to cope
197 with large variations in nutrient composition, with respect to sugar content, nitrogen content,
198 in oxygen supply and in other parameters such as low or high pH, presence of anti-nutritional
199 factors, etc.

200 What's more, we also observed various duration of lag phase of different strains
201 (Supplementary Table 1). Although the lag phase can be avoided by seed cultivation in
202 industry, the shortest lag phase duration of *B. subtilis* NCIB3610 in both AS and AN medium
203 suggested that it's more adapted to the nutrient-limited environments than other strains.
204 Given those advantages with the high growth rate, *B. subtilis* NCIB3610 seemed to be the
205 most promising host for aquaculture wastewater denitrification.

206 On the other hand, DSM1092 and some *B. subtilis* 168 legacy strains (Fig. 1A), like WB800,
207 and 168S showed difficulty in growing on media with sole inorganic nitrogen source. But their
208 growth was obviously improved by addition of protein and amino acids especially when DO
209 level was increased (Fig. 3C, Fig. 3D).
210 However, DSM1092 in this study exhibited rapid depletion of overflow compounds (acetate
211 and acetoin) in MEG medium without an intact glyoxylate shunt reported (Fig. 4A). This
212 characteristic is beneficial for industrial applications, such as growth on additional carbon
213 sources and better robustness towards excess glucose in environment.
214 Based on the growth properties of strains, we hypothesize that a) Strains containing part of
215 genome sequence of *B. subtilis* W23 are more adapted to different nutritional environments
216 (Fig. 1B). b) *B. subtilis* DSM1092 and some legacy strains of 168 have domesticated
217 metabolic pathways for organic nitrogen sources utilization rather than ammonium salts
218 because there are always sufficient organic nitrogen sources in their culture environment.

219 Pellicle formation

220 The biofilm matrix of *B. subtilis* has been extensively studied for various personal care
221 applications such as biocatalyst for therapeutic Menaquinone-7 production or as protector for
222 beneficial probiotics[18][19]. Among all selected variants, *B. subtilis* NCIB3610 and PS832
223 formed clear pellicles in media containing organic nitrogen source at first day (Supplementary
224 Fig. 1), suggesting that *B. subtilis* NCIB3610 and PS832 are more suitable for biofilm matrix
225 producing than others. On the other hand, biofilm can be a disadvantage during liquid state
226 fermentation, as it affects the transfer of nutrients and oxygen in the system and increase the
227 difficulty of fermenter cleaning. In this regard, *B. subtilis* 168S is a better choice to avoid the
228 biofilm matrix during fermentation as its biofilm formation capacity may be disturbed by
229 deletion of *spo0A*.

Food additives

Current protein production for feed and food, involving various plant and animals, is highly inefficient, has major environmental impact with respect to land/water-use and Greenhouse gas emissions and often involves non-local production resulting in a need for long-distance transportation. However, the microbes offer a more efficient approach to obtaining protein as they are able to grow fast (hours to double their biomass), have innately high protein content and can be produced locally with limited land- and water-usage. In our case, all spore-deficient variants (*B. subtilis* 3NA, 168S and PY79S) showed higher protein proportion than others (~37% by dry cell weight) while the famous spore producer - PY79 showed the lowest protein ratio. These findings suggested that the spore-deficient *B. subtilis* strains can serve as good microbial protein producers.

Diacetyl is a flavour responsible for buttery aroma and is widely used in the food and beverage industry[20]. It can be transformed spontaneously from acetoin in the acetolactate pathway, so extracellular acetoin profiles can reflect the capacity of Diacetyl production of *B. subtilis* variants. Based on metabolite profiles, we observed that optimal DO conditions were conducive to the expression of acetolactate pathway while high dissolved DO promoted biomass gaining. Given the growth and metabolites profile in AS medium, we proposed that the non-sporulate mutants (especially PY79S) might be good hosts for potential products within the acetolactate pathway such as diacetyl because they had shorter fermentation time, hence, higher productivity.

Bio-based polymers

Over past decades, the increasing demand for “green materials” have opened new windows of opportunity for bio-based products like bio-based polymers that is more environmental-friendly than petroleum-derived consumables.

Dipicolinic acid, Lactic acid and 2,3-Butanediol are promising monomers which can be harnessed by fermentation and can be used for polymeric materials production. Researchers have been developing different microorganism to covert sugars into DPA. For example, Lynn et al. established a DPA synthesis pathway by overexpression of dipicolinate synthase genes from *Paenibacillus sonchi* in a *Corynebacterium glutamicum* L-lysine producer strain, resulting in 2.5 g/l of DPA in shake-flask[21]. K. McClintock et al. achieved a titer of 4.7 g/l DPA using an *E. coli* strain by knocking out competitive pathways and heterogeneous expressing dipicolinate synthase from *B. subtilis*[22]. Compared with abovementioned bacteria, *B. subtilis* possesses complete DPA synthesis pathway naturally as DPA is a vital compound in *B. subtilis* spores for heat resistance. In addition, *B. subtilis* can also produce L-lactic acid which is a bulk chemical and can be polymerized to produce biodegradable polymer PLA for food packaging[23]. Combining the growth and metabolite profiles of *B. subtilis* strains under different DO conditions, we recommended WB800 and PS832 as hosts for DPA production under aerobic conditions, PY79S as promising host for 2.3-Butanediol and lactate production under aerobic conditions and oxygen-deprived condition, respectively.

Conclusion

In this study, we first investigated the growth flexibility of various *B. subtilis* strains under different conditions, such as growth rates and cellular components. Then, we elucidated the effects of environment on the metabolism of *B. subtilis* strains, such as the distribution of metabolites in the acetolactate pathway, lactate synthesis pathway and acetate synthesis pathway in different periods. Based on results, we recommend some *B. subtilis* strains that are uncommonly used in the development of cell factories, such as DSM1092 and 3NA for further development of cell factories. All in all, we conclude that *B. subtilis* is an underdeveloped cell factory with great metabolic flexibility under different nutrient conditions

278 and various aeration regimes. By understanding its metabolic potential under various
279 conditions, we can expand the use of *B. subtilis* beyond enzyme production.

280 Material and methods

281 Strains and media

282 All strains used in this study are listed in Table 4. As shown in Fig. 1A, *B. subtilis* NCIB3610
283 is the closest descendant of the lab strain *B. subtilis* Marburg and shares a similar genotype
284 with the widely used transformation-efficient *B. subtilis* 168 (Fig. 1B). However, they have
285 obvious differences in phenotypes like substrate utilization, metabolites synthesis and swarm
286 ability due to the mutations at *trpC*, *swrA*, *sfp* and *gudB* loci. *B. subtilis* WB800, a derivative
287 of *B. subtilis* 168, lacks eight extracellular proteases that helps to stabilize secreted proteins.
288 *B. subtilis* PY79, 3NA and PS832 possess some genomic characteristics of *B. subtilis* W23
289 and have been commonly used for sporulation and germination research. The prototroph *B.*
290 *subtilis* PY79 possesses 4.3% reduction in genomic size compared with *B. subtilis* 168, and
291 34 kb replacement of *B. subtilis* 168 genes with orthologous *B. subtilis* W23 genes. *B. subtilis*
292 3NA produces no spores due to a frame-shift mutation at *spo0A* locus and an elongation at
293 *abrB* gene which both affects the initialization of sporulation. The *B. subtilis* PS832 is seemed
294 as the “prototrophic and revertant” version of *B. subtilis* 168. It has been used to exploit spore
295 germination like germinant receptors in Setlow’s lab and our lab for a long period. To address
296 the potential demand for blocking sporulation, we also constructed two spore-deficient strains
297 named *B. subtilis* 168S and *B. subtilis* PY79S in this study, whose *spo0A* gene was entirely
298 knocked out. In addition to the family of lab strains, we selected one food-grade *B. subtilis*
299 DSM1092 that is isolated from traditional Japanese food Natto for research .Overall, we
300 selected nine representative *B. subtilis* strains to explore their metabolic potential for cell
301 factory development.

Table 4 Strains used in this study

Strains	Description (; source)	Reference
B. subtilis NCIB3610	Wild type; BGSC[a]	[14]
B. subtilis 168	*trpC2*; Lab stock	[10]
B. subtilis PY79	Prototroph SPβ[s]; Lab stock	[12]
B. subtilis DSM1092	Wild type; DSMZ[b]	[16]
B. subtilis WB800	*trpC2, nprE, aprE, epr, bpr, mpr*::*ble nprB*::*bsr* Δ*vpr wprA*::*hyg*; Lab stock	[17]
B. subtilis 3NA	*spo0A3*; BGSC	[11]
B. subtilis PS832	Prototroph; Lab stock	[15]
B. subtilis 168S	*B. subtilis* 168 Δ*spo0A* :: Cm^R; Lab stock	This study
B. subtilis PY79S	*B. subtilis* PY79 Δ*spo0A* :: Cm^R; Lab stock	This study
E. coli MG1655	F^-, λ^-, *rph-1*; Lab stock	[24]

[a]*BGSC* Bacillus genetic stock center; [b]*DSMZ* Leibniz institute DSMZ-German collection of microorganisms and cell cultures GmbH

The Luria-Bertani (LB) medium was used for strain propagation and consisted of 5 g/l Yeast extract, 10 g/l Tryptone, and 5 g/l NaCl. For seed culture and microbial protein fermentation, LBG was adapted with the addition of 1‰ 50%(w/v) glucose in LB medium. All fermentation media contained the same basal salts solution: 12.5 g/l $K_2HPO_4 \cdot 3H_2O$, 3 g/l KH_2PO_4, 3 g/l $MgSO_4 \cdot 7H_2O$, 10 ml/l Trace elements solution. Trace elements solution consists of 4 g/l $FeSO_4 \cdot 7H_2O$, 4 g/l $CaCl_2$, 1 g/l $MnSO_4 \cdot 5H_2O$, 0.04 g/l $CoCl_2 \cdot 6H_2O$, 0.2 g/l $NaMoO_4 \cdot 2H_2O$, 0.2 g/l $ZnSO_4 \cdot 7H_2O$, 0.1 g/l $AlCl_3 \cdot 6H_2O$, 0.1 g/l $CuCl_2 \cdot H2O$, 0.05 g/l H_3BO_4. For strain cultivation in Meat extract (ME) medium, 9 g/l Meat extract was supplemented as the only nitrogen source. Similarly, 9 g/l $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, peptone and casein hydrolysate were added as nitrogen sources in Ammonium sulfate (AS) medium, Ammonium nitrate (AN) medium, Ammonium chloride (ACl) medium, Peptone (PE) medium and Casein hydrolysate (CA) medium, respectively. By supplementing ME, PE and CA with an additional 6 g/l glucose, we made MEG, PEG and CAG media, respectively. In addition, AS, AN and ACl medium were supplemented with 6 g/l glucose as the carbon source. Particularly, 0.05 g/l of tryptophan was added in the media for 168, WB800 and 168S as they are auxotroph mutants.

319 To wash strains, a 10 x PBS solution was prepared with 35.81 g/l $Na_2HPO_4.12H_2O$, 2.45 g/l
320 KH_2PO_4, 80.07 g/l NaCl, 2.01 g/l KCl.

321 Construction of spore-deficient strains

322 The primers and DNA templates used for gene amplification are listed in Supplementary table
323 2. The homogenous arms of *spo0A*-gene were amplified from *B. subtilis* 168 and the
324 chloramphenicol resistance gene was amplified from plasmid pDG1662 according to the
325 manufacturer's instruction (Takara Primestar max DNA polymerase). These three fragments
326 were combined by overlapping Polymerase Chain Reaction (PCR) and purified by DNA
327 purification kit (Thermofisher). The purified product was then transformed into *B. subtilis* 168
328 and PY79 by the method of Anagnostopoulos & Spizizen, resulting in the 168S and PY79S
329 strains[25].

330 Seed culture for fermentation

331 Cultures of strains were taken from glycerol stocks stored at -80 °C and were streaked on LB
332 medium plates which were then placed in a 37 °C incubator overnight. For each strain, one
333 colony was picked into a 50 ml Erlenmeyer flask loaded with 12 ml of LB medium. After 6
334 hours of incubation, the cultures were centrifuged at 10,000 rpm for 2 minutes, and the
335 resulting pellets were washed three times with 1 × PBS. Finally, the pellets were resuspended
336 in 1 × PBS to obtain OD of 10.

337 Cultivation experiments in 96-well plate and flask

338 For the fermentation experiments in 96-well plates, each well was loaded with 150 μl medium
339 and inoculated with a washed seed culture (1% (v/v)). The inoculated microplates were
340 covered with oxygen-permeable films and inserted into the microplate reader for 48 h growth
341 curve measurement at 37°C.

342 For the fermentation experiments in flasks, different dissolved oxygen (DO) conditions were
343 created by loading different amount of fermentation broth in Erlenmeyer flasks or glass
344 bottles[26]: 50 ml/250 ml flask (High DO condition), 100 ml/250 ml flask (optimal DO condition),
345 and 18 ml/20 ml bottle (oxygen-deprived condition). After inoculation, the flasks were
346 incubated in a shaking incubator at 37°C, 200 rpm for 72 hours. The bottles were placed
347 static in a 37°C incubator. Samples were taken at 4, 8, 12, 24, 36, 48 and 72 hours.

348 Dry cell weight and protein concentration measurement

349 The LBG medium was selected for strain propagation and the measurement of biomass was
350 performed as follows. A single colony was picked up from LB medium plate into a flask
351 containing 50 ml liquid LB medium with 50 μl glucose (50% solution) and placed in the
352 shaking incubator at 37°C. After 6 and 24 hours, the cells were harvested and centrifuged at
353 10000 rpm for 2 minutes. After three washes with PBS solution, the OD_{600} of suspended
354 culture was measured. The culture was then diluted or condensed to create a 2 ml
355 suspension in PBS solution or Ambic water (ammonium bicarbonate with 1% SDS) with an
356 OD of 4 for dry cell weight and protein concentration, respectively.
357 A piece of filter paper was used to filter 2ml of cell suspension with an OD of 4 in PBS. After
358 filtering all the liquid, the filter papers were placed back in the stove at 95°C. For dry cell
359 weight measurement, 10 pieces of filter paper (0.22 μm) were thoroughly dried in a 95°C
360 stove and their weight was determined up to four decimal places. A piece of filter paper was
361 used to filter 2ml of the cell suspension of OD = 4 in PBS. After all the liquid was filtered, the
362 filter papers were placed back in the stove at 95°C. After every 24 hours of drying, the papers
363 were weighed until the weight showed no change. For microbial protein measurement, the
364 cells were lysed with glass beans (0.1 mm) and the tubes were centrifuged at 15,000 rpm for
365 10 minutes. Protein concentration of *B. subtilis* cells was measured using a Thermo

366 Scientific™ Pierce™ BCA protein assay. All steps followed the instruction on the assay kit
367 manual.

368 Dry spore weight and Dipicolinic acid measurement

369 All strains were revived on LB agar plates and incubated at 37°C overnight. Single colony
370 was picked up into 50 ml 3-(N-morpholino) propane sulfonic acid medium (MOPS) buffered
371 defined liquid medium (Sigma-Aldrich, St. Louis, MI, USA). For DSM1092, which could not
372 propagate in the MOPS medium, 1% (v/v) LB medium was added to the mixture. Pure spores
373 of strains were harvested after 3-day incubation in a shaking incubator at 37°C, 200 rpm. The
374 MOPS medium and spore purification method was as described previously by Tu and co-
375 workers [27]. Spore dry weights were determined by weighing overnight freeze-dried spores
376 with an OD of 10. To measure DPA in each sample, 2 OD_{600} of spore was resuspended with
377 a buffer consisting of 0.3 mM $(NH_4)_2SO_4$, 6.6 mM KH_2PO_4,15 mM NaCl, 59.5 mM $NaHCO_3$
378 and 35.2 mM Na_2HPO_4. Subsequently, the suspended spores were incubated at 100°C for 1
379 hour with a control set incubated at 37°C for 1 hour. After incubation, samples were
380 centrifuged at 15,000 rpm for 2 minutes and 10μl of the supernatant was then added to 115μL
381 of another buffer (1 mM Tris, 150 mM NaCl) with and without 0.8 mM terbium chloride (Sigma-
382 Aldrich, St. Louis, MI, USA) for a 15-min incubation at 37°C. The fluorescence of the samples
383 was measured by a Synergy Mx microplate reader (BioTek; 270-nm excitation; 545-nm
384 reading; gain, 100) (Bad Friedrichshall, Germany). The background fluorescence (without
385 terbium, incubated at 37°C) was subtracted from that of all the samples. A calibration curve
386 of 0–140 mg/l DPA was used to calculate DPA concentrations of the sample.

387 Extracellular metabolites measurement

388 The supernatant samples were centrifuged at 15,000 rpm for 10 min and filtered by 0.22 μm
389 filters. The standards for the High-Performance Liquid Chromatography (HPLC)
390 measurement of oxoglutarate, fumarate, malate, lactate, acetate, acetoin, glucose and

391 ethanol were prepared with a set of concentration within 0-50 mM. All standards and samples
392 were measured by HPLC using a Shimadzu instrument (LC-20AT, Prominence, Shimadzu)
393 equipped with an Ion exclusion Rezex ROA-Organic Acid H+(8%) column (300 × 7.8 mm
394 column, Phenomenex), along with a guard column (Phenomenex). Aqueous H_2SO_4 (5 mM)
395 was used as mobile phase at a flow rate of 0.5 ml/min at 55 °C. A wavelength of 210 nm was
396 used for calibration and analysis with the SPD-20A UV/VIS detector, and a cell temperature
397 of 40°C was set for refractive index detector (RID 20A, Shimadzu).

398 ## Statistical Analysis

399 All data are presented as the means ± standard deviation (SD). Three biological replicates
400 were measured for biomass and spore weight measurement. Statistical analyses like
401 Ordinary One-way ANOVA (Tukey) were carried out using OriginPro 2022 software and
402 Prism GraphPad software.

403 # Abbreviations

404 ***AS medium:*** *Fermentation medium with ammonium sulfate as sole nitrogen source*

405 ***AN medium:*** *Fermentation medium with ammonium nitrate as sole nitrogen source*

406 ***ME medium:*** *Fermentation medium with meat extract as sole nitrogen source*

407 ***MEG medium:*** *ME medium with extra glucose*

408 ***DO:*** *Dissolved oxygen*

409 ***DPA:*** *Dipicolinic acid*

410 ***HPLC:*** *High-Performance Liquid Chromatography*

411 # Declarations

412 ## Ethics approval and consent to participate

413 Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization, JH, SB and TC; performing experiments and analyzing data, TC; drafting original manuscript, TC; reviewing and editing, JH and SB. All authors read and approved the final manuscript.

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