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/excretion and sporulation and as model bacterium for controlling spoilage in the food industry. It has also been used for production of several secondary metabolites such as (commercial) enzymes and 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 unconventional 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 (biobased) 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 *E. coli*[1], *B. subtilis*[2] and *S. 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]. Based on that, different *B.*

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

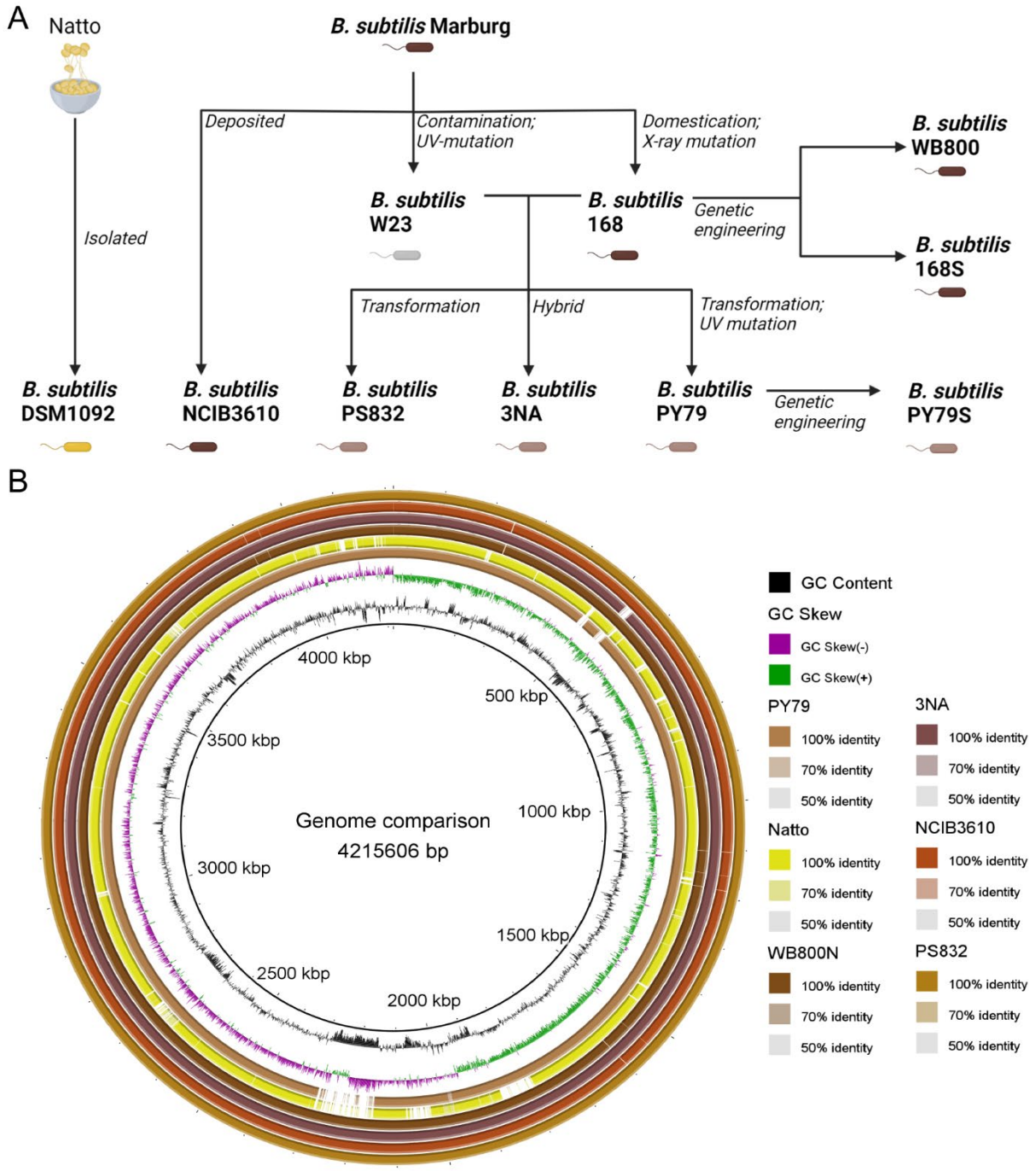


Figure 1 | A. Genome heritage of nine *B. subtilis* strains. B. Genomic sequence comparison of representative *B. subtilis* strains using genome of 168 as the reference.

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

80 growth rates (μ) and growth curves in Fig. 2. *B. subtilis* NCIB3610, PY79S, PS832, 3NA and
81 PY79 exhibited higher specific growth rates than other strains in media with ammonium salts
82 (Fig. 2A, Fig. 2B), suggesting that they are competitive in these environments. Especially, *B.*
83 *subtilis* NCIB3610 showed the highest growth rate of 0.33 h^{-1} and 0.35 h^{-1} among all strains
84 in media with ammonium sulfate and with ammonium nitrate, respectively (Fig. 2A, Fig. 2B).
85 Subsequently, we replaced the ammonium salts and glucose with meat extract (ME medium)
86 to assess the growth characteristics of *B. subtilis* strains in the environment containing free
87 amino acids, peptides and proteins. In ME medium, different strains showed similar specific
88 growth rates. Notably, the growth rate of DSM1092 increased from 0.06 h^{-1} to 0.38 h^{-1} after
89 switching the nitrogen source from inorganic to organic, suggesting that it is adapted for the
90 environment containing organic nitrogen source(Fig. 2C). By further adding glucose into ME
91 medium (MEG medium), we observed that the growth rate of DSM1092 increased by 28.9%
92 from 0.38 h^{-1} to 0.49 h^{-1} (Fig. 2D).
93 In addition to growth rates, *B. subtilis* strains exhibited different growth patterns when growing
94 in different media. In the media containing organic nitrogen sources, most strains showed
95 immediate propagation and started to decline after reaching the peak, whereas the downward
96 trend was not the same for different strains (Fig. 2C, Fig. 2D). In media with ammonium salts,
97 all strains presented obvious lag phases (Supplementary table 1) and long stationary phase
98 when growing in AS medium(Fig. 2A). Unlike the abovementioned cases, strains showed
99 both different lag phases and a decreasing trend after reaching the highest biomass in AN
100 medium (Fig. 2B).

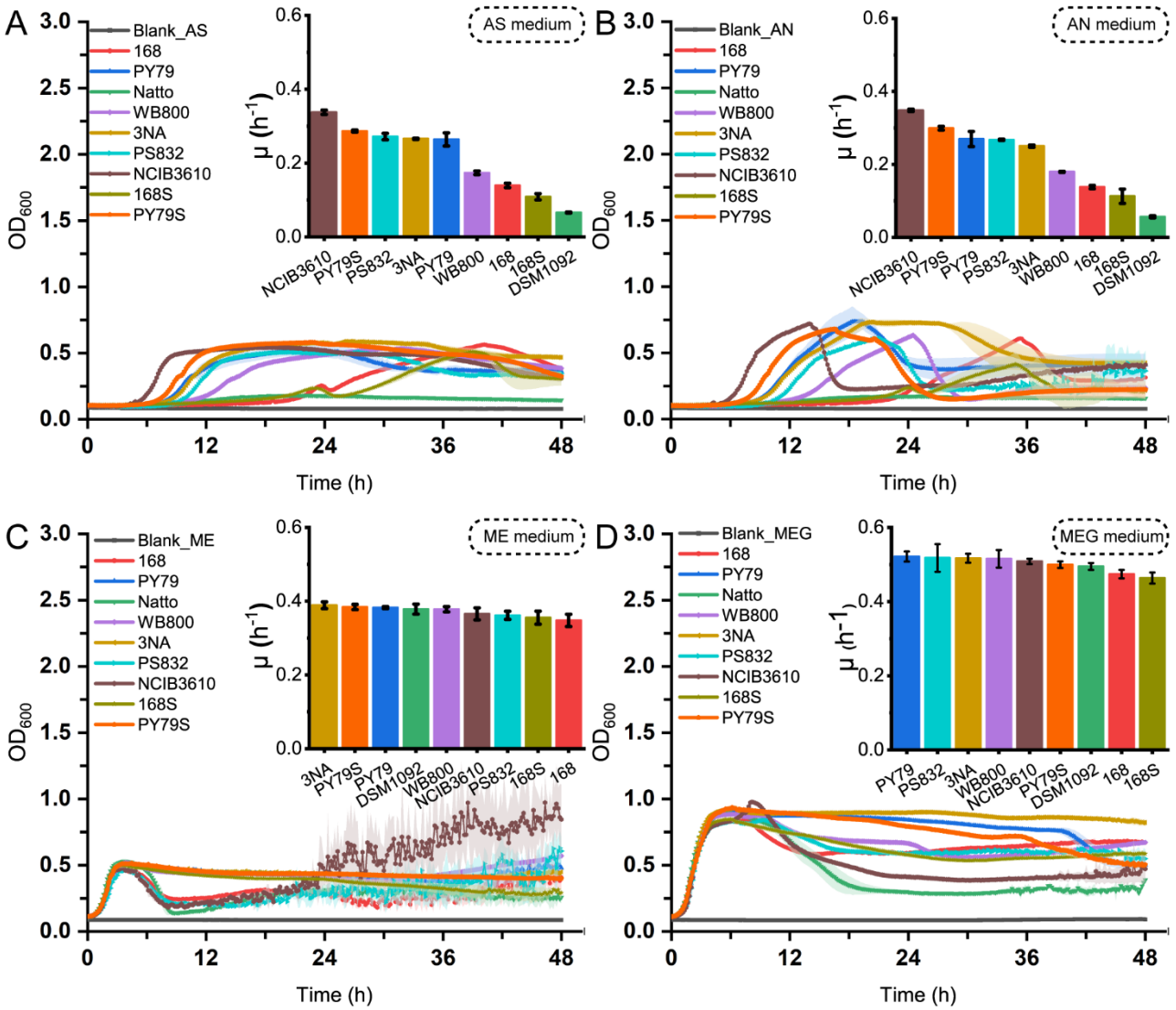


Figure 2 | Growth curves and specific growth rates of nine *B. subtilis* strains on various media. A. Growth properties of nine strains in medium with ammonium sulphate; B**.** Growth properties of nine strains in medium with ammonium nitrate; C**.** Growth properties of nine strains in medium with meat extract; D. Growth properties of nine strains in medium with meat extract and glucose. The shallow surrounding growth curve represents the standard deviations of replicates.

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

To evaluate the effect of oxygen on *B. subtilis* growth, we created high Dissolved oxygen (DO), optimal DO and oxygen-deprived conditions for fermentation experiments in flask and measured OD_{600} values of strains in 72h. When ammonium salt was used as the only nitrogen source, only the growth of NCIB3610 showed an obvious improvement with increase of

112 dissolved oxygen(Fig. 3A, Fig. 3B). However, in the medium containing free amino acids, all
113 strains grew faster with the increase of dissolved oxygen. For example in MEG medium, the
114 OD_{600} value of *B. subtilis* DSM1092 reached 15.97, which was 3.1 times the value under
115 optimal DO condition (Fig. 3D). Furthermore, as the protein and polypeptide content in the
116 medium decreased, the influence of dissolved oxygen on the growth pattern of the strain
117 became less. When casein hydrolysate was the only organic nutrient in the medium, elevating
118 dissolved oxygen had no effect on the growth of all strains (Supplementary Fig. 2C).
119 Additionally, we observed that *B. subtilis* strains only grew in the presence of both glucose
120 and nitrate or both glucose and organic nitrogen sources under oxygen-deprived condition.
121 However, NCIB3610 reached 0.54 in AN medium, far exceeding other variants (Fig. 3B).
122 Moreover, 3NA and PY79 whose highest OD_{600} were both 1.4, outpaced other strains by
123 faster growth and higher biomass in MEG medium (Fig. 3D).

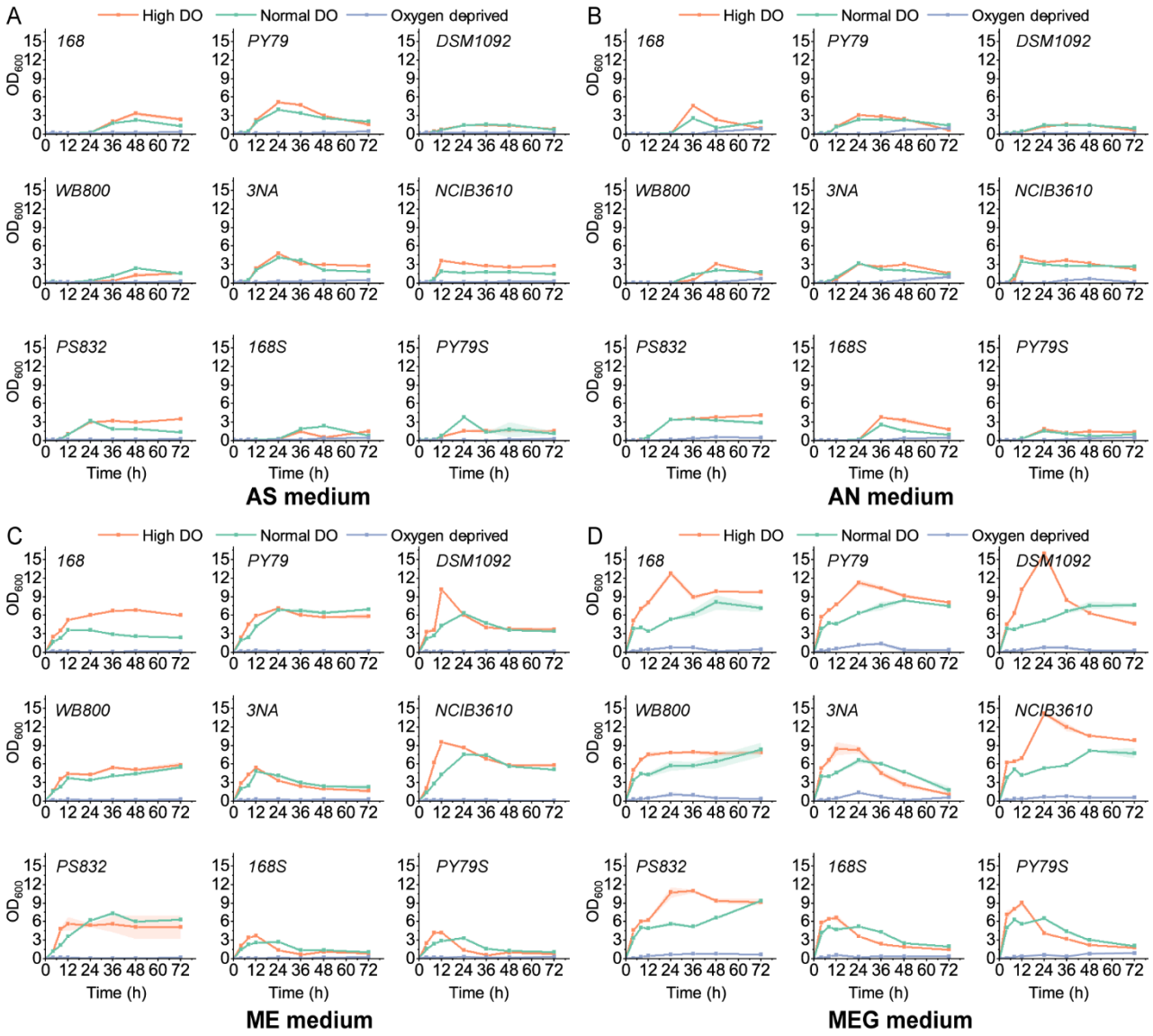


Figure 3 | A~D. Growth curves of strains in flasks in medium with ammonium sulphate (AS), ammonium nitrate (AN), meat extract (ME) and both meat extract and glucose (MEG) under high DO, optimal DO and oxygen-deprived conditions. The shallow surrounding growth curve represents the standard deviations 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 with LBG medium in 250-ml flasks for 24h. At 6h (exponential phase), *B. subtilis* strains showed similar dry cell weight and protein proportions, while the protein ratios of strains

134 varied from 10.35% to 37.54% at 24h (stationary phase) (Table 1). At 24h, spore-deficient *B.*
135 *subtilis* 3NA exhibited the highest proportion of 37.54%, 3.4 times higher than that of PY79
136 which is a well-known spore-forming variant. The protein proportion of PY79 also increased
137 from 10.6% to 36% after we disrupted its sporulation via knocking out *spo0A* (PY79S),
138 indicating the negative effect of sporulation on microbial protein proportion.

Table 1 Dry cell weight and protein concentration of strains

Strains	6h			24h		
	Dry weight (g/l/OD)	**Protein concentration (g/l/OD)**	**Proportion (%)**	**Dry weight (g/l/OD)**	**Protein concentration (g/l/OD)**	**Proportion (%)**
MG1655	0.530	0.089	16.792	0.288	0.113	39.236
168	0.415	0.048	11.566	0.425	0.044	10.353
PY79	0.390	0.057	14.615	0.500	0.053	10.600
DSM1092	0.355	0.068	19.155	0.538	0.082	15.242
WB800	0.400	0.058	14.500	0.400	0.123	30.750
3NA	0.415	0.068	16.386	0.325	0.122	37.538
NCIB3610	0.440	0.066	15.000	0.260	0.066	25.385
PS832	0.435	0.073	16.782	0.210	0.038	18.095
168S	0.460	0.072	15.652	0.588	0.166	28.231
PY79S	0.420	0.051	12.143	0.475	0.171	36.000

139 Although *B. subtilis* spores are usually discussed with food spoilage and contamination, they
140 are also related to dipicolinic acid (DPA) production which is industrial relevant. To determine
141 spore dry weight and DPA content in the spores of *B. subtilis* strains, we cultivated strains in
142 MOPS medium in 250-ml flasks for 72h, and purified spores. As a result, we didn't find DPA
143 in supernatant of cultures but only in spores. *B. subtilis* 168 stored 74.2 mg of DPA in 1 g of
144 dry spore, which is the lowest among selected strains (Table 2). However, two mutants
145 WB800 and PS832 derived from 168 showed the highest DPA production, namely 122.9 mg
146 and 131.0 mg of DPA in 1 g of dry spore, respectively.

Table 2 Spore properties of strains

Strains	Dry weight (g/l/OD)	DPA concentration (mg/l/OD)	Proportion (mg_{DPA}/g_{spore})
168	0.09	6.68	74.2
PY79	0.11	10.22	92.9
DSM1092	0.13	7.56	58.2
WB800	0.10	12.29	122.9
NCIB3610	0.10	8.77	87.7
PS832	0.05	6.55	131.0

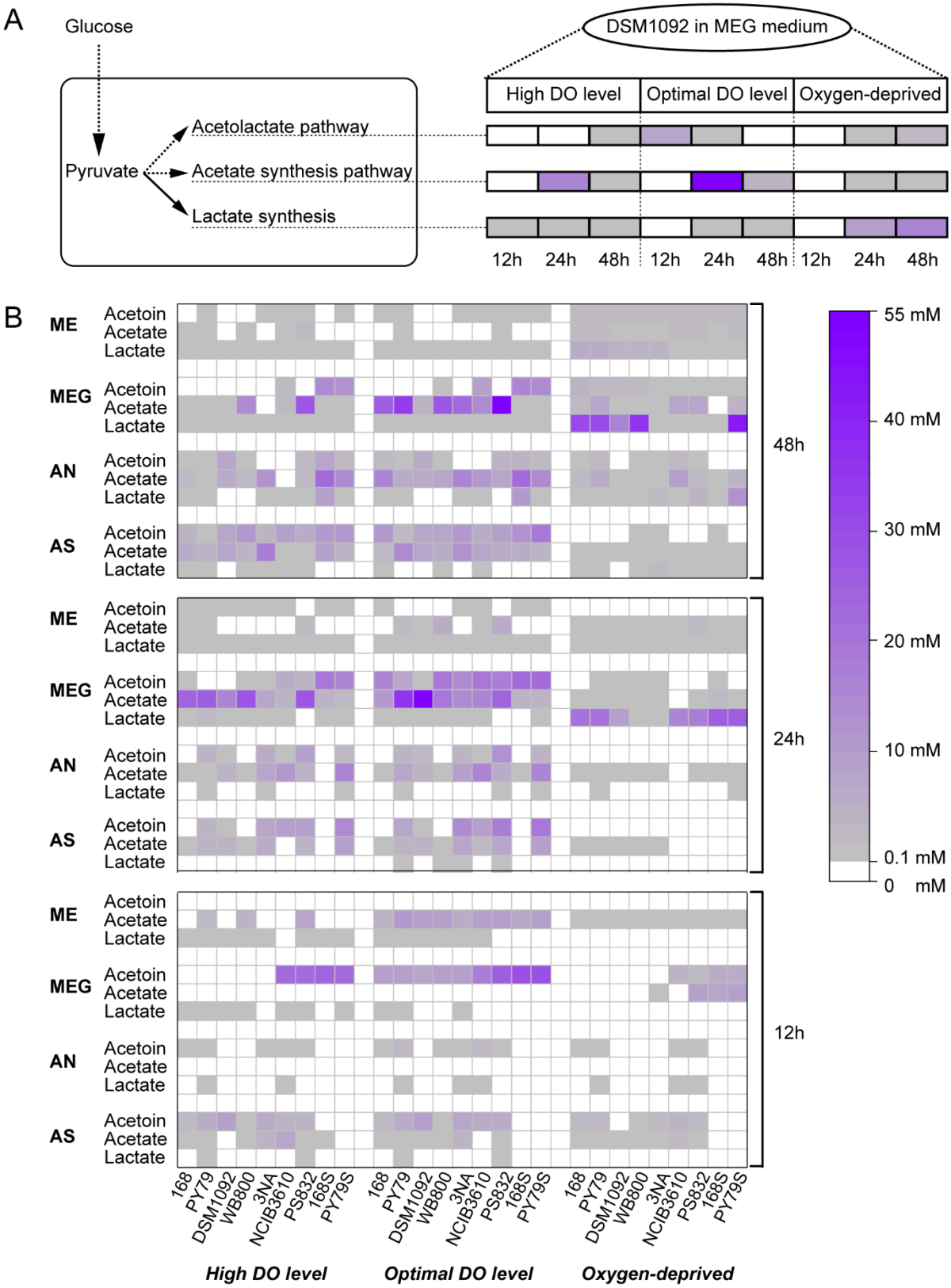
147 ## Variation in extracellular metabolites distribution of *B. subtilis* strains

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

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

164 Then we assessed the acetate synthesis pathway. The most interesting finding was two
165 different patterns of acetate production between *B. subtilis* DSM1092 and PS832 in MEG
166 medium under aerobic conditions. Acetate concentrations in the supernatants of PS832

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



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177 **Figure 4** | A. Metabolite profile of DSM1092 in MEG medium. B: Metabolic distribution of nine *B. subtilis* strains

178 in medium with ammonium sulphate (AS), ammonium nitrate (AN), meat extract (ME) and both meat extract

179 and glucose (MEG) under high DO, optimal DO and oxygen-deprived conditions.

180 ## Discussion

181 In this study, we investigated metabolic flexibility of different *B. subtilis* strains corresponding
182 to various industrial applications and recommended certain variants for specific usage (Table
183 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

184 ### Biorefinery

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

191 While determining growth rates of *B. subtilis* strains in different media, we also observed
192 various duration of lag phase of different strains (Supplementary Table 1). Although the lag
193 phase can be avoided by seed cultivation in industry, the shortest lag phase duration of *B.*
194 *subtilis* NCIB3610 in both AS and AN medium suggested that it's more adapted to the
195 nutrient-limited environments than other strains. Given that with the high growth rate, *B.*
196 *subtilis* NCIB3610 seemed to be the most promising host for aquaculture wastewater

197 denitrification. On the other hand, DSM1092 and some *B. subtilis* 168 legacy strains (Fig. 1A),
198 like WB800, and 168S showed difficulty in growing on media with sole inorganic nitrogen
199 source. But their growth was obviously improved by addition of protein and amino acids
200 especially when DO level was increased (Fig. 3C, Fig. 3D). What's more, DSM1092 exhibited
201 rapid depletion of C-2 compounds (acetate and acetoin) in MEG medium without an intact
202 glyoxylate shunt reported (Fig. 4A). This characteristic is beneficial for industrial applications,
203 such as growth on additional carbon sources and better robustness towards excess glucose
204 in environment.
205 Based on the growth properties of strains, we hypothesize that a) Strains containing part of
206 genome sequence of *B. subtilis* W23 are more adapted to different nutritional environments
207 (Fig. 1B). b) *B. subtilis* DSM1092 and some legacy strains of 168 have domesticated
208 metabolic pathways for organic nitrogen sources utilization rather than ammonium salts
209 because there is always sufficient organic nitrogen sources in their culture environment.

210 Pellicle formation

211 The biofilm matrix of *B. subtilis* has been extensively studied for various personal care
212 applications such as biocatalyst for therapeutic Menaquinone-7 production or as protector for
213 beneficial probiotics[11][12]. Among all selected variants, *B. subtilis* NCIB3610 and PS832
214 formed clear pellicles in media containing organic nitrogen source at first day (Supplementary
215 Fig. 1), suggesting that *B. subtilis* NCIB3610 and PS832 are more suitable for biofilm matrix
216 producing than others. On the other hand, biofilm can be a disadvantage during liquid state
217 fermentation, as it affects the transfer of nutrients and oxygen in the system and increase the
218 difficulty of fermenter cleaning. In this regard, *B. subtilis* 168S is a better choice to avoid the
219 biofilm matrix during fermentation as its biofilm formation capacity may be disturbed by
220 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[13]. 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 higher productivity and were less to worry about in terms of intermediate retaking.

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.

245 Dipicolinic acid, Lactic acid and 2,3-Butanediol are promising monomers which can be
246 harnessed by fermentation and can be used for polymeric materials production. Researchers
247 have been developing different microorganism to covert sugars into DPA. For example, Lynn
248 et al. established a DPA synthesis pathway by overexpression of dipicolinate synthase genes
249 from *Paenibacillus sonchi* in a *C. glutamicum* L-lysine producer strain, resulting in 2.5 g/l of
250 DPA in shake-flask[14]. K. McClintock et al. achieved a titer of 4.7 g/l DPA using an *E. coli*
251 strain by knocking out competitive pathways and heterogeneous expressing dipicolinate
252 synthase from *B. subtilis*[15]. Compared with abovementioned bacteria, *B. subtilis* possesses
253 complete DPA synthesis pathway naturally as DPA is an vital compound in *B. subtilis* spores
254 for heat resistance. In addition, *B. subtilis* can also produce L-lactic acid which is a bulk
255 chemical and can be polymerized to produce biodegradable polymer PLA for food
256 packaging[16]. Combining the growth and metabolite profiles of *B. subtilis* strains under
257 different DO conditions, we recommended WB800 and PS832 as hosts for DPA production
258 under aerobic conditions, PY79S as promising host for 2.3-Butanediol and lactate production
259 under aerobic conditions and oxygen-deprived condition, respectively.

260 Conclusion

261 In this study, we first investigated the growth flexibility of various *B. subtilis* strains under
262 different conditions, such as growth rates and cellular components. Then, we elucidated the
263 effects of environment on the metabolism of *B. subtilis* strains, such as the distribution of
264 metabolites in the acetolactate pathway, lactate synthesis pathway and acetate synthesis
265 pathway in different periods. Based on results, we recommend some unconventional *B.*
266 *subtilis* strains such as DSM1092 and 3NA for further development of cell factories. All in all,
267 we conclude that *B. subtilis* is an underdeveloped cell factory with great metabolic flexibility
268 under different nutrient conditions and various aeration regimes. By understanding its

269 metabolic potential under various conditions, we can expand the use of *B. subtilis* beyond
270 enzyme production.

271 Material and methods

272 Strains and media

273 All strains used in this study are listed in Table 4. As shown in Fig. 1A, *B. subtilis* NCIB3610
274 is the closest descendant of the lab strain *B. subtilis* Marburg and shares a similar genotype
275 with the widely used transformation-efficient *B. subtilis* 168 (Fig. 1B). *B. subtilis* WB800, a
276 derivative of *B. subtilis* 168, lacks eight extracellular proteases that helps to stabilize secreted
277 proteins. *B. subtilis* PY79, 3NA and PS832 possess some genomic characteristics of *B.*
278 *subtilis* W23 and have been commonly used for sporulation and germination research. To
279 address the potential demand for blocking sporulation, we also chose two spore-deficient
280 strains named *B. subtilis* 168S and *B. subtilis* PY79S. In addition to the family of lab strains,
281 we selected one food-grade *B. subtilis* DSM1092 that is isolated from traditional Japanese
282 food Natto for research .Overall, we selected nine representative *B. subtilis* strains to explore
283 their metabolic potential for cell factory development.

Table 4 Strains used in this study

Strains	Description (; source)	Reference
B. subtilis NCIB3610	Wild type; BGSC[a]	[17]
B. subtilis 168	*trpC2*; Lab stock	[18]
B. subtilis PY79	Prototroph SPβ^{s}; Lab stock	[19]
B. subtilis DSM1092	Wild type; DSMZ[b]	[20]
B. subtilis WB800	*trpC2, nprE, aprE, epr, bpr, mpr*::*ble nprB*::*bsr* Δ*vpr wprA*::*hyg*; Lab stock	[21]
B. subtilis 3NA	*spo0A3*; BGSC	[22]
B. subtilis PS832	Prototroph; Lab stock	[23]
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]

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

286 The Luria-Bertani (LB) medium was used for strain propagation and consisted of 5 g/l Yeast
287 extract, 10 g/l Tryptone, and 5 g/l NaCl. For seed culture and microbial protein fermentation,
288 LBG was adapted with the addition of 1‰ 50%(w/v) glucose in LB medium. All fermentation
289 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
290 $MgSO_4 \cdot 7H_2O$, 10 ml/l Trace elements solution. Trace elements solution consists of 4 g/l
291 $FeSO_4 \cdot 7H_2O$, 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 $NaMoO_4 \cdot 2H_2O$,
292 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
293 cultivation in Meat extract (ME) medium, 9 g/l Meat extract was supplemented as the only
294 nitrogen source. Similarly, 9 g/l $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, peptone and casein hydrolysate
295 were added as nitrogen sources in Ammonium sulfate (AS) medium, Ammonium nitrate (AN)
296 medium, Ammonium chloride (ACl) medium, Peptone (PE) medium and Casein hydrolysate
297 (CA) medium, respectively. By supplementing ME, PE and CA with an additional 6 g/l glucose,
298 we made MEG, PEG and CAG media, respectively. In addition, AS, AN and ACl medium
299 were supplemented with 6 g/l glucose as the carbon source. Particularly, 0.05 g/l of
300 tryptophan was added in the media for 168, WB800 and 168S as they are auxotroph mutants.
301 To wash strains, a 10 x PBS solution was prepared with 35.81 g/l $Na_2HPO_4.12H_2O$, 2.45 g/l
302 KH_2PO_4, 80.07 g/l NaCl, 2.01 g/l KCl.

303 Seed culture for fermentation

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

Cultivation experiments in 96-well plate and flask

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

For the fermentation experiments in flasks, different dissolved oxygen (DO) conditions were created by loading different amount of fermentation broth in Erlenmeyer flasks or glass bottles[25]: 50 ml/250 ml flask (High DO condition), 100 ml/250 ml flask (optimal DO condition), and 18 ml/20 ml bottle (oxygen-deprived condition). After inoculation, the flasks were incubated in a shaking incubator at 37°C, 200 rpm for 72 hours. The bottles were placed static in a 37°C incubator. Samples were taken at 4, 8, 12, 24, 36, 48 and 72 hours.

Dry cell weight and protein concentration measurement

The LBG medium was selected for strain propagation and the measurement of biomass was performed as follows. A single colony was picked up from LB medium plate into a flask containing 50 ml liquid LB medium with 50 μl glucose (50% solution) and placed in the shaking incubator at 37°C. After 6 and 24 hours, the cells were harvested and centrifuged at 10000 rpm for 2 minutes. After three washes with PBS solution, the OD_{600} of suspended culture was measured. The culture was then diluted or condensed to create a 2 ml suspension in PBS solution or Ambic water (ammonium bicarbonate with 1% SDS) with an OD of 4 for dry cell weight and protein concentration, respectively.

A piece of filter paper was used to filter 2ml of cell suspension with an OD of 4 in PBS. After filtering all the liquid, the filter papers were placed back in the stove at 95°C. For dry cell weight measurement, 10 pieces of filter paper (0.22 μm) were thoroughly dried in a 95°C stove and their weight was determined up to four decimal places. A piece of filter paper was used to filter 2ml of the cell suspension of OD = 4 in PBS. After all the liquid was filtered, the

335 filter papers were placed back in the stove at 95°C. After every 24 hours of drying, the papers
336 were weighed until the weight showed no change. For microbial protein measurement, the
337 cells were lysed with glass beans (0.1 mm) and the tubes were centrifuged at 15,000 rpm for
338 10 minutes. Protein concentration of *B. subtilis* cells was measured using a Thermo
339 Scientific™ Pierce™ BCA protein assay. All steps followed the instruction on the assay kit
340 manual.

341 Dry spore weight and Dipicolinic acid measurement

342 All strains were revived on LB agar plates and incubated at 37°C overnight. Single colony
343 was picked up into 50 ml 3-(N-morpholino) propane sulfonic acid medium (MOPS) buffered
344 defined liquid medium (Sigma-Aldrich, St. Louis, MI, USA). For DSM1092, which could not
345 propagate in the MOPS medium, 1% (v/v) LB medium was added to the mixture. Pure spores
346 of strains were harvested after 3-day incubation in a shaking incubator at 37°C, 200 rpm. The
347 MOPS medium and spore purification method were as described previously by Tu and co-
348 workers [26]. Spore dry weights were determined by weighing overnight freeze-dried spores
349 with an OD of 10. To measure DPA in each sample, 2 OD_{600} of spore was resuspended with
350 a buffer consisting of 0.3 mM $(NH_4)_2SO_4$, 6.6 mM KH_2PO_4,15 mM NaCl, 59.5 mM $NaHCO_3$
351 and 35.2 mM Na_2HPO_4. Subsequently, the suspended spores were incubated at 100°C for 1
352 hour with a control set incubated at 37°C for 1 hour. After incubation, samples were
353 centrifuged at 15,000 rpm for 2 minutes and 10μl of the supernatant was then added to 115μL
354 of another buffer (1 mM Tris, 150 mM NaCl) with and without 0.8 mM terbium chloride (Sigma-
355 Aldrich, St. Louis, MI, USA) for a 15-min incubation at 37°C. The fluorescence of the samples
356 was measured by a Synergy Mx microplate reader (BioTek; 270-nm excitation; 545-nm
357 reading; gain, 100) (Bad Friedrichshall, Germany). The background fluorescence (without
358 terbium, incubated at 37°C) was subtracted from that of all the samples. A calibration curve
359 of 0–140 mg/l DPA was used to calculate DPA concentrations of the sample.

360 ## Extracellular metabolites measurement

361 The supernatant samples were centrifuged at 15,000 rpm for 10 min and filtered by 0.22 μm
362 filters. The standards for the HPLC measurement of oxoglutarate, fumarate, malate, lactate,
363 acetate, acetoin, glucose and ethanol were prepared with a set of concentration within 0-50
364 mM. All standards and samples were measured by HPLC using a Shimadzu instrument
365 equipped with an 300 × 7.8 mm column (Phenomenex), a UV detector and a refractive index
366 detector. Aqueous H_2SO_4 (5 mM) was used as mobile phase at a flow rate of 0.5 ml/min at
367 55 °C.

368 ## Statistical Analysis

369 All data are presented as the means ± standard deviation (SD). Three biological replicates
370 were measured for biomass and spore weight measurement. Statistical analyses were
371 carried out using OriginPro 2022 software.

372 **Abbreviations**

373 ***DO:*** *Dissolved oxygen*

374 ***DPA:*** *Dipicolinic acid*

375 **Declarations**

376 ## Ethics approval and consent to participate

377 Not applicable.

378 ## Consent for publication

379 Not applicable.

380 ## Availability of data and materials

381 The datasets used and/or analyzed during the current study are available from the
382 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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