Biodecomposing *Spirulina platensis* by a *de novo* designed *Bacillus subtilis*-based method to develop a medium for the high cell-density cultivation of *Escherichia coli* in batch mode

Xiaowen Li,[a, b] Zhengqi Wei,[b] Jingyi Ge,[b] Yingying Pan,[b] Xiang Chu,[b] Baolin Huang,[b] Jiawei Zhao,[b] Yanzhao Li,[b] Yizhuang Zhou[b#]

[a] Institute of Pathogeny Biology, School of Basic Medicine, Guilin Medical University, Guilin, Guangxi 541199, China

[b] Guangxi Key Laboratory of Environmental Exposomics and Entire Lifecycle Health, School of Public Health, Guilin Medical University, Guilin, Guangxi 541199, China

Running Head: MSP for *E. coli* HCDC

[#]Address correspondence to Yizhuang Zhou, zhouyizhuang3@163.com.

Word count for abstract: 250 (for abstract) + 150 (for importance)

Word count for the text: 5463 (including materials and methods)

Abstract

High cell-density cultivation (HCDC) is fundamental to basic research and industrial applications, especially in batch mode. However, limited media are available for batch culture of HCDC, because the media for batch culture must contain extremely sufficient nutrients on the one hand and few or even no substrates to generate detrimental metabolite on the other hand to attain HCDC. *Spirulina platensis* (SP), a new media material, is considered ideal for the development of such media. Here, we develop a biolysis method for SP degradation based on the cultivation supernatant of *Bacillus subtilis* and extensively demonstrate its higher degradation (indicated by the production of more small peptides and free amino acids) and cultivation effectiveness with three other methods. Based on its SP hydrolysates, a modified SP-based broth (MSP) is then formulated. Tests on *Escherichia coli* K-12 show that MSP achieves HCDC with several benefits: (*i*) its maximum optical density at 600 nm is ~16.67, significantly higher than that of Luria-Bertani (LB) broth (only ~6.30); (*ii*) MSP requires only 36 h to reach peak growth, much faster than that of LB (48 h); (*iii*) its maximum growth (1.12 ± 0.01 h^{-1}) is significantly higher than that of LB (0.20 ± 0.00 h^{-1}); (*v*) MSP initiates growth immediately after inoculation (lag time <0), comparable to LB; (*iv*) the number of viable cells in MSP is high (~2.16×10^{11} ml^{-1}), ~10.19 times the amount in LB. Consequently, we envision MSP will become the first choice for *E. coli* HCDC batch culture in the future.

Importance

So far, it is the first time to develop a high-efficiency method for transforming *Spirulina platensis* (SP) into medium ingredients. Based on its SP hydrolysates, a high cell-density cultivation (HCDC) medium for the batch culture of *Escherichia coli* is formulated for the first time, which is greatly beneficial for both basic research and industrial applications. In addition to HCDC, the SP hydrolysates can be extended to a wide range of applications, due to their rich nutrient content. Besides, this study demonstrates for the first time that SP is an ideal material to develop HCDC media. Furthermore, this study demonstrates that medium development and modification for batch culture can attain HCDC, without the development of new culture technologies. Therefore, this study highlights the importance of the rebirth of medium development and modification and supports the shift from developing new culture technologies to medium development and modification for HCDC in batch mode.

Keywords: *Spirulina platensis*; high cell density cultivation; biodegradation; growth medium; batch culture.

Introduction

Cell growth is fundamental to both basic research and industrial application, requiring bacteria to grow to high cell density to maximize biomolecules including plasmids and proteins, especially for the pioneer bacterium *Escherichia coli*. To achieve high cell-density cultivation (HCDC), requisite nutrients should be sufficiently supplied, and toxic or inhibitory factors generated by metabolism amid the cultivation should be avoided or removed meanwhile (1). Under this logic, some promising culture

technologies, including fed-batch culture (2, 3), dialysis culture (4-6), and semicontinuous culture (7), had been developed and applied accordingly, attaining HCDC *via* providing additional nutrients and diluting or removing inhibitors coincidentally. However, they all require sophisticated instrumentation and some of them such as dialysis culture waste a considerable amount of nutrients, combinedly leading to high cost. Therefore, they are not routinely used in laboratories and industries. In contrast, batch culture is preferred in laboratories and even in industry, owing to simple handling, high feasibility, easy parallelization, and reduced investment in equipment. To attain HCDC for batch culture, the media should contain extremely sufficient nutrients on one hand, as all necessary nutrients are provided once before culture in batch mode; on the other hand, the media should contain few or even no substrates to generate detrimental metabolites, due to no removal or dilution of detrimental metabolites during the whole cultivation. Accordingly, it is difficult for a medium to satisfy both requirements together. Therefore, to the best of our knowledge, such a medium is not yet available. For example, the widely-used rich Luria-Bertani (LB) medium can only reach saturation of ~7 optical density at 600 nm (OD600) for *E. coli* (8); glucose, one most favorable carbon source for *E. coli* (9), inhibits growth above the concentration of 50 g/l (10) or by its metabolite acetate acid (11). In this sense, developing a novel medium for HCDC is of pronounced importance and urgent.

Spirulina platensis (SP), a member of the Phormidiaceae family with rich nutrients (12), is an ideal material to potentially develop culture media for HCDC based on the

following considerations. First, it is one of the richest protein sources with a protein content of around 60-70% (13), greatly higher than that in egg, beer yeast, skimmed powdered milk, fish, soy, and beef meat (14), some of which are traditional sources for media. Second, the most abundant monosaccharide in SP is rhamnose, which accounts for 53% of the total sugars (15), while glucose is relatively low (<2%) (16); this is beneficial to prevent/decrease acetate acid generation, especially by excess glucose (17). Third, SP contains all essential minerals and trace elements (14, 18), as well as luxuriant bioavailable vitamins including vitamins A, E, B1, B7, and B8 (14). Finally, SP contains numerous antioxidant ingredients, including phycocyanin, carotenoids, and xanthophylls (19), which may be beneficial to ameliorate oxidant stress under aerobic conditions. Besides, SP, as a photoautotrophic microorganism, can be cultivated on a large scale in a small area at low cost and absorbs atmospheric CO_2, making a positive impact on the environment (20). Therefore, SP was here selected to develop an HCDC medium for *E. coli*.

So far as we know, little or almost no research has been reported on transforming SP into medium ingredients (20, 21), hampering its utilization for HCDC or even routine cultivation. The difficulty of its transformation may suffer from the two following reasons. First, SP cell is difficult to disrupt due to their high resistance, especially for dry SP (22). Second, SP proteins should be further degraded into small peptides or even free amino acids for HCDC, as most bacteria can only utilize free amino acids due to the scarcity of peptide transporters (23). Even for *E. coli* with several oligopeptide

permeases and peptidases (8), efficient degradation is also beneficial, as free amino acids can be directly utilized to accelerate growth, with small peptides instead of large peptides utilized after complete degradation. *Bacillus subtilis* is commonly found in the soil. Provided that nutrients are sparse or changing in soil, *B. subtilis* has to produce extracellular proteases to support its growth and survive *via* protein degradation. So far, at least eight secreted proteases have been reported (24). Except for the cell wall-associated protease (WprA) (25), the remaining proteases are secreted into the growth medium, potentially supplying an opportunity to efficiently degrade SP by using the cultivation supernatant of *B. subtilis*.

Here, we developed a *B. subtilis*-based method to lyse SP and demonstrated that this method outperforms the three mechanical methods in terms of small peptides obtained, amino acids liberated, and growth performance achieved. Based on the products of this method, as a test for *E. coli* K-12, we further developed a modified SP-based medium (hereinafter named MSP) and found that MSP achieved HCDC (OD600 of ~16.97) rapidly within 36 h, with a significantly larger maximum growth rate and ~10.19 times the number of viable cells in comparison to the reference LB medium. To the best of our knowledge, it is the first time for us to biodegrade SP to achieve HCDC in batch mode. We envisage that MSP developed here will become the medium of first choice for *E. coli* HCDC in batch culture in the future.

Results

Development of biolysis methods using varying cultivation supernatant of *B.*

subtilis

As all the 7 aforementioned extracellular proteases secreted into cultivation supernatant are highly produced during the plateau growth phase (24), the cultivation supernatant sampled at 72 h was used for SP degradation, based on the growth kinetics of *B. subtilis* determined here (Fig. S1). It was observed that *B. subtilis* growth was significantly enhanced in Nutrient Broth (NB) than in LB (Fig. S1), which may provide more extracellular proteases in NB than in LB to increase SP degradation. However, both NB and LB cultivation supernatants were explored to see which one was indeed better. To further determine the optimal volume, aliquots of 1 ml, 3 ml, and 9 ml of cultivation supernatants were added into 300 ml sterilized SP solutions, each of which contained 45 g SP dry power with initial pH at ~7 and was autoclaved at 121℃ for 15 min to eliminate the effect of microorganisms on SP consumption. After 5 h degradation, we found that the pH levels of all SP solutions were decreased (Fig. S2A), possibly due to the two highest-abundance acidic amino acids liberated into the solutions, namely glutamic acid (~8.386%) and aspartic acid (~5.793%) (14), which were confirmed by the results below (Fig. 4a). Among them, the pH fall was the largest in the product of the method with 3 ml NB cultivation supernatant (Fig. S2A), with a significant difference with the products of others except by using 9 ml NB cultivation supernatant, indicating that 3 ml NB cultivation supernatant may have the best lysis. To confirm it, a direct formol titration method (26) was applied. Expectedly, our results showed that 3 ml NB cultivation supernatant has the significantly best degradation effectiveness (Fig.

S3A).

Considering that proteases may partially reduce or even lose their activities under acidic conditions, we wondered whether the addition of fresh cultivation supernatant could increase SP degradation. To this end, 4 extra successive addition-degradation cycles were performed. Due to pH falls, SP products were neutralized with 1 M NaOH to eliminate their effects on degradation, and then added with 1.893 g of KH_2PO_4 and 2.803 g of K_2HPO_4 (0.1 M potassium phosphate, pH 7.0) to provide buffering capacity to postpone pH falls. Subsequently, fresh cultivation supernatant with respective volume was added and then subjected to 5-h degradation. It was observed that the pH levels of all SP products were always decreased after each 5-h degradation and the processing with 3 ml NB cultivation supernatant regularly yielded the largest pH falls, albeit with or without significant difference compared to other processing (Figs. S2B-2E). Subsequent formol titration assays repeatedly demonstrated that 3 ml NB cultivation supernatant significantly outperformed all other treatments (Figs. S3B-3D). After 25-h degradation, the processing with 3 ml NB cultivation supernatant achieved the best degradation performance (Fig. 1A).

Comparison of growth performance on original SP broths as culture media

After the above 25-h biodegradation, we accordingly obtained six different original SP broths. To further determine which one was best, a quantitative comparison of cultivation was conducted *via* testing *E. coli* K-12. It was observed that the best growth was significantly achieved for the broth derived from the degradation by using 3-ml NB

163 cultivation supernatant regardless of the sampled times (Fig. 1B), with a significantly
164 highest maximum growth rate (Fig. 1C) and a significantly or insignificantly reduced
165 lag time (Fig. 1D). Also, this broth attained a significantly highest maximum OD600
166 predicted by *grofit* (Fig. 1E) and obtained a statistically highest integral value on the
167 whole (Fig. 1F). Consequently, based on all findings (Fig. 1), the above processing with
168 3 ml NB cultivation supernatant was determined as the protocol of the biolysis method
169 developed in this study and the resultant original SP broth (OSP) was obtained for
170 following experiments.

171 **Superiority in degradation and cultivation effectiveness compared with other**
172 **methods**

173 Although there are several conventional methods available to disrupt SP cells for the
174 extract of phycocyanin (27), little or almost no methods are available to degrade SP for
175 developing the SP-based media. It is worth mentioning that the methods used for the
176 extract of phycocyanin are relatively different from the SP degradation in this study, as
177 the former involves gentle cell disintegration to obtain intact phycocyanin, while the
178 latter involves vigorous SP degradation into short peptides and even free amino acids.
179 However, in an attempt to demonstrate the conspicuousness of our method, we
180 compared the biolysis method with three conventional methods including alternate
181 freezing and thawing (28), sonication (29), and water bath.

182 For comparison of degradation effectiveness, formol titration assays were carried out.
183 Our findings showed that the released amino acids are greatly significantly higher in the

184 products of our biolysis method than in the products of the three conventional methods
185 ($P < 0.0001$, Fig. 2A), demonstrating that our method is more effective. To further
186 verify its superiority, cultivation trials on *E. coli* K-12 were performed and monitored
187 from 12 h to 120 h. It was observed that the cell densities reached in OSP generated by
188 our biolysis method were significantly higher than those in other SP broths during the
189 whole incubation period, with an averaged maximum OD600 of ~14.92 at 72 h, while
190 only approaching 10.36, 6.90, and 6.90 for alternate freezing and thawing, sonication
191 and water bath at 60 h respectively (Fig. 2B). *E. coli* K-12 was cultivated with a
192 maximum growth rate of 0.3130 ± 0.0131 h^{-1}, significantly higher ($P < 0.001$) than that
193 in the SP broths generated by the alternate freezing and thawing (0.2355 ± 0.0017 h^{-1}),
194 sonication (0.1772 ± 0.0012 h^{-1}) and water bath (0.1780 ± 0.0017 h^{-1}) respectively (Fig.
195 2C). However, its lag time was significantly longer than those by sonication and water
196 bath, but insignificantly shorter than that by alternate freezing and thawing (Fig. 2D),
197 possibly illustrating why the time to reach maximum growth is longer than those by
198 other methods (Fig. 2B). The maximum OD600 predicted by *grofit* was $15.0072 \pm$
199 0.2075, 10.1892 ± 0.0686, 8.1229 ± 0.1071, and 8.0278 ± 0.0780 for SP broths
200 generated by the methods of biolysis, alternate freezing and thawing, sonication and
201 water bath respectively (Fig. 2E). Finally, our biolysis method yielded a significant
202 higher integral value than others (Fig. 2F). Taken together, these findings demonstrated
203 that our biolysis method outperforms other methods mentioned here to achieve HCDC
204 by testing *E. coli* K-12, albeit with a significantly or insignificantly longer lag time.

205 **Demonstration of increased degradation effectiveness by gel electrophoresis**

206 If SP is effectively decomposed and then efficiently degraded, SP proteins should be
207 greatly released and transformed into short peptides or even free amino acids afterward.
208 Accordingly, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
209 was conducted to compare the degradation effectiveness in terms of short peptides
210 between the 4 methods. As shown in Fig. 3A, protein degradation was evidenced by gel
211 smearing in the SDS-PAGE portrait. Our biolysis method seemed to display increased
212 degradation effectiveness in relation to other methods, as visualized by the more
213 degraded proteins accumulated at the bottom of the gel (Fig. 3A). To further quantify
214 the degradation, densitometry by ImageJ was conducted. It was observed that the area
215 intensity for <10 kDa accounted for ~42.92% of the total intensities for the biolysis
216 method, which was significantly higher than that for other methods (Fig. 3B). Also, the
217 area intensities for the ranges of 10-15 kDa (Fig. 3C) and 15-20 kDa (Fig. 3D) were
218 conspicuously statistically higher for the biolysis method than for other methods
219 respectively. Conversely, the intensity for the remaining areas was greatly lower than
220 that by other methods with a statistical difference ($P < 0.01$, Fig. 3E). In conclusion,
221 these findings authenticated that the biolysis method achieved higher degradation
222 effectiveness with respect to other methods. This is very beneficial for some bacteria
223 such as *Lactobacillus amylovorus* (30), which prefers peptide-bound amino acids to free
224 amino acids, and *E. coli* with several oligopeptide permeases and peptidases (8).

225 **Precise comparison of released amino acids by ultra-high performance liquid**

chromatography-tandem mass spectrometry (UHPLC-MS/MS)

It is noteworthy that the formol titration method utilized above is rough to quantify released amino-acids, due to the coexistence of phosphates, polypeptides, fatty acids, and carbohydrates, whose reactions are also acid or alkaline to eventually affect the titration endpoint (31). To reliably measure the contents of released amino acids, UHPLC-MS/MS, which was demonstrated to be rapid, specific, and sensitive for profiling free amino acids (32, 33), was accordingly applied. All 20 amino acids except cysteine were detected in all SP products (Fig. 4A). Glutamic acid, the highest-abundance (~8.386%) amino acid in SP (14), was highly abundant (951.03 ± 109.54 ug/ml) in the SP products of all 4 methods without statistical differences (Fig. 4A), while the second most abundant (~5.793%) aspartic acid in SP (14), the other acidic amino acid, was liberated in relatively lower amounts, suggesting that the aforementioned pH falls (Fig. S2) were possibly due to the liberated glutamic acid mainly and aspartic acid partially. Nonetheless, the biolysis method released about 2.80, 3.01, and 3.64 times the amount of aspartic acid liberated by alternate freezing and thawing, water bath, and sonication respectively. Serine, the amino acid reported to be consumed first (34), was insignificantly liberated by the 4 methods. The releases of all remaining amino acids except tyrosine and arginine were significantly higher in our biolysis method than in other methods (Fig. 4A). However, arginine was reported to be a poor nitrogen source (9), and the medium without tyrosine alone or in combination with cysteine was reported to achieve denser culture than medium with all 20 amino

247 acids (35), accounting for the growth conspicuousness of SP broth generated by the
248 biolysis method than those by the other 3 methods (Fig. 2B and 2E). Collectively, the
249 level of total amino acids released by the biolysis method was significantly higher in
250 comparison to other methods (Fig. 4B), in line with the findings of formol titration
251 assays (Fig. 2A), supporting that the biolysis method degrades SP more effectively than
252 the 3 other methods.

253 **Further improved growth performance by formulating OSP into MSP**

254 To further increase growth performance, a systematic investigation on OSP modification
255 was conducted and finally, the formulated MSP was obtained to contain 15% (w/v) OSP,
256 2.524 g of KH_2PO_4, and ~3.737 g of K_2HPO_4 (potassium phosphate buffer at pH 7.0,
257 PPB7.0, totaling 0.04 M), 1% (w/v) glucose, and 2.03 mM $MgSO_4$. It is noteworthy that
258 the OSP became gradually alkaline over the course of the cultivation (Fig. 5A), possibly
259 due to the excretion of the ammonia derived from the consumption of amino acids in
260 OSP (36). On the contrary, an initial drop of the pH down to 6.6 was observed in MSP
261 (Fig. 5A), possibly due to acid generation by glucose metabolism (37), as glucose was
262 preferentially catabolized *via* carbon catabolic repression (38); then the pH increases
263 gradually up to a maximum of 7.2 at 120 h (Fig. 5A), possibly due to the buffering
264 capacity of PPB7.0 against alkalization generated by amino acid metabolism (Fig. S4A)
265 (36). Together, MSP maintained pH in the best range of 6.5-7.5 as mentioned previously
266 for *E. coli* cultivation (39), which is very important for microbial growth.

267 Growth comparison showed that MSP increases the maximum OD600 from ~14.92 to

268 ~16.67 (Fig. 5B), with a significant difference demonstrated by *grofit* extraction (Fig.
269 5E), possibly due to the addition of both PPB7.0 partially (Fig. S4B) and 2.03 mM
270 $MgSO_4$ mainly (Fig. S6A and Fig. S6D). This is because $MgSO_4$ supplementation in the
271 millimolar range harbors promoting effect on growth (35), and PPB7.0 maintained pH
272 in the best range of 6.5-7.5 (39). Interestingly, the time to reach the maximum OD600
273 was reduced from 72 h to 36 h, attributing to a significantly increased maximum growth
274 rate (Fig. 5C) in combination with a statistically reduced lag time (Fig. 5D). The
275 increased maximum growth rate was due to the supplementation of PPB7.0 (Fig. S4C),
276 1% glucose (Fig. S5B), and 2.03 mM $MgSO_4$ (Fig. S6B), and the reduced lag time was
277 due to the amendment of 1% glucose (Fig. S5C), while PPB7.0 even increased the lag
278 time (Fig. S4D). Glucose is one of the most favorable carbon sources to support the fast
279 growth of *E. coli* when ammonia rather than a single poor nitrogen source such as
280 arginine, glutamate, or proline is used as a nitrogen source (9). Our OSP has plentiful
281 amino acids (Fig. 4) to generate sufficient ammonia, while glucose was negligible in all
282 SP products of the 4 degradation methods due to below the analytical detection limit of
283 UHPLC-MS/MS (data not shown). Therefore, glucose fuels growth and reduces lag
284 time. However, the mechanism underlying why PPB7.0 increased both the maximum
285 growth rate and the lag time awaits further investigation. Therefore, the integral value of
286 MSP was significantly larger than that of OSP for the growth curves until 72-h
287 cultivation (Fig. 5F), with the aim of achieving HCDC here.

288 **Outstanding growth performance in comparison to LB**

For comparison, *E. coli* K-12 was cultivated in the reference medium LB. We found that MSP achieved a high averaged maximum growth (OD600) of ~16.67, whilst only ~ 6.30 for LB (Fig. 6A). Besides, MSP required only 36 h to reach maximum growth, much faster than LB which required 48 h (Fig. 6A), which is greatly beneficial for both basic research and industrial application. Extraction of 4 growth parameters showed that MSP achieved a significantly higher maximum growth rate (Fig. 6B), initiated growth immediately after inoculation (indicated as lag time <0 h) albeit with a significantly longer lag phase (Fig. 6C), and reached a higher maximum growth of 1.12 ± 0.01 h^{-1} (Fig. 6D) and integral (Fig. 6E), compared with LB. Collectively, MSP is superior to LB, attaining HCDC in batch culture.

It was reported that cells in HCDC contain viable cells, viable but not culturable cells, cells under lysis, and dead cells (40). Among them, viable cells are more valuable. Therefore, viable cells were counted by plating cultures sampled at 36 h. Remarkably, MSP attained 2.16×10^{11} ml^{-1} of viable cells, ~10.19 times the amount obtained by LB (Fig. 6F), while their OD600 fold is only ~3.08 (16.67/5.42) (Fig. 6A). This indicated that MSP is more suitable to produce/maintain viable cells than LB even in the exponential growth phase. In conclusion, MSP attains HCDC with high viability.

Discussion

Many effects have been made to search for new sources for preparing media (20, 41-43). However, none of them achieved HCDC. SP, which was demonstrated as a novel material for media preparation (20), was considered by us here as an ideal material to

potentially develop culture media for HCDC (see detailed reasons, please refer to the "Introduction" section); this hypothesis was demonstrated in this study. To the best of our knowledge, it is the first time for us to demonstrate that SP can be utilized to achieve HCDC in batch mode for *E. coli* (20). Besides, so far, SP is the first material that can be developed to attain batch HCDC for *E. coli*.

To reach HCDC, investigations had been shifted from batch culture to several promising culture technologies, including fed-batch culture (2, 3), dialysis culture (4-6), and semicontinuous culture (7). However, these systems were not preferable in laboratories and even in industries, due to the requirement of sophisticated instrumentation or great waste of nutrients (dialysis culture as an example). In contrast, this study showed that medium development and modification can achieve HCDC in batch mode, highlighting the importance of the rebirth of medium development and modification.

OSP has plentiful free amino acids (Fig. 4) and short peptides (Fig. 3). Amino acids including both free and peptide-bound amino acids can be used as single carbon sources (44) and key substrates for achieving a high specific growth rate for *E. coli* (45), while their metabolisms result in considerably less acetate acid (46), which is detrimental for growth (11). Also, it is noteworthy that OSP contains negligible glucose, due to below the analytical detection limit of UHPLC-MS/MS (data not shown), avoiding the generation of acetate acid. The excellent growth performance (~14.92 OD600) of OSP obtained in this study indicates that metabolism optimization *via* supplying "good"

media can reach HCDC in batch mode, further supporting the rebirth of medium development and modification for HCDC in batch mode and the shift from developing new culture technologies to medium development and modification for HCDC in batch mode.

Finally, it is the first time for us to report a biolysis method with high efficiency for SP here (20). Based on its SP hydrolysates, MSP was formulated in this study and attained HCDC for *E. coli*. However, we realize that OSP or MSP with rich nutrients developed here could be extended to other applications, including recombinant protein production, DNA transformation, rehydration of lyophilized cells (20) and rapid resuscitation of lactic acid-induced sublethal injuries (47). Consequently, the biolysis method as well as OSP or MSP are fundamental, due to their great potential for wide application. Further studies should focus on exploring their extended applications.

Materials and Methods

Growth condition and growth kinetics for *B. subtilis*

Each aliquot of 1 ml *B. subtilis* fresh pre-cultures (~1.00 OD600) in Nutrient Broth (NB) was inoculated into 10 ml fresh NB or LB and cultivated for 72 h (37℃, 200 rpm). To establish growth kinetics, OD600 measurement was periodically recorded by a UV–2700 spectrophotometer (Shimadzu, Japan) in triplicate at 12-h intervals. After 72-h cultivation, the supernatants of *B. subtilis* in ~9-ml quantities were collected *via* centrifugation at 10,000 g for 20 min and preserved at 4℃ for downstream SP degradation.

352 **SP degradation by cultivation supernatant of *B. subtilis***

353 SP dry powder of 45 g was soaked in 300 ml distilled water and then autoclaved at
354 121℃ for 15 min to eliminate the effect of microorganisms on SP consumption. Our
355 checking found that the initial SP solutions were at pH ~7. After cooling down, 1 ml, 3
356 ml, and 9 ml of preserved NB or LB cultivation suspension of *B. subtilis* were added. At
357 5-h intervals, samples were collected for downstream formol titration and their pH
358 levels were electrometrically monitored offline with a pH electrode (PHS-3C, INESA
359 Scientific Instrument Co., Ltd, Shanghai, China). To eliminate the effect of different pH
360 falls on SP degradation, all resulting SP solutions were adjusted to pH 7.0 with 1 M
361 NaOH. Then, 1.893 g of KH_2PO_4 and 2.803 g of K_2HPO_4 (totaling 0.1 M potassium
362 phosphate, pH 7.0) were jointly added to provide buffering capacity, followed by the
363 addition of fresh cultivation supernatant of *B. subtilis* with the same volumes. After 4
364 successive addition-degradation cycles, the final products were centrifuged at 10,000 g
365 for 20 min to obtain their aqueous supernatants. Then, to compensate for the volume
366 loss, an appropriate amount of water was added to respective samples, which were
367 subsequently neutralized by 1 M NaOH and finally sterilized by autoclaving at 121℃
368 for 15 min and preserved at 4℃ for the following experiments.

369 **Degradation procedures of the mechanical methods**

370 Dried SP power was soaked in distilled water at 15% (w/v), autoclaved at 121℃ for 15
371 min, and subjected to processing by the following reference methods. For the sonication
372 method, the SP solution was sonicated with a sonotrode of 2 mm diameter and 100%

power input in 8 × 5 min periods without any cooling breaks in between. For the water bath method, the SP solutions were preheated at 60℃ for 30 min to avoid the break of the flask followed by a bath at 100℃ for 60 min. For the alternate freezing and thawing method, the SP liquids were processed by four freezing (-20℃) and thawing (room temperature) cycles. After processing, all samples were centrifuged at 10,000 g for 20 min, precipitates were discarded and the aqueous supernatants were sterilized by autoclaving at 121℃ for 15 min for the following experiments.

Rough measurement of SP protein hydrolysis by formol titration

An Aliquot of 2 ml above sampled SP degradation solution after centrifugation was measured out with a pipette into each of two 100-ml Erlenmeyer flasks. To each Erlenmeyer flask added 5 ml of distilled water. One of the Erlenmeyer flasks served as a color screen and the other one severed as the tested sample. Besides, a third Erlenmeyer flask containing only 7 ml distilled water was used as a formalin blank. Five drops of the color indicator Phenolphthalein and then 2 ml of formalin were added into each of the three Erlenmeyer flasks. After amply twirling, a burette of 0.1 M NaOH was used for titration until the pink color of the indicator was visible. The titration of a sample subtracted by the titration of the formalin blank was then used to indicate the protein hydrolysis of this sample. In total, three biological replicates were conducted for each sampled SP degradation solution.

Gel electrophoresis and densitometry by ImageJ

The polyacrylamide gel was composed of a stacking gel (30% acrylamide in 1.00 M

Tris-HCl buffer, pH 6.8) and a 15% acrylamide separating gel in 1.5 M Tris-HCl buffer, pH 8.8. The supernatants obtained above were used to compare the degradation efficiency of the 4 methods. SDS-PAGE was performed under reducing conditions at 60 V through the stacking gel and 80 V in the separation gel until the tracking dye migrated to the bottom of the gel. Gel was stained in a solution of 1% (w/v) Coomassie blue R-250, 40% (v/v) methanol, and 16.6% (v/v) acetic acid, and decolorized under continuous shaking in a 25% (v/v) ethanol solution containing 10% (v/v) acetic acid. A low-molecular-weight pre-stained protein ladder (Affinity Biosciences Group Ltd., Jiangsu, China) composed of ten recombinant protein bands of 250, 150, 100, 70, 50, 40, 35, 25, 20, 15, and 10 kDa was used to indicate the molecular weight. Gels were scanned with AlphaImager HP Gel Imaging System (Alpha Innotech Corp., USA) under UV epi-illumination. Images were saved as a PNG file with a size of 16-bit and a resolution of 1,228 × 884 pixels and then converted to 8-bit grayscale images using the ImageJ (Sun Microsystems Inc., USA) for densitometric quantification. After background subtraction, the area intensities of <10 kDa, 10-15 kDa, 15-20 kDa, and >20 kDa were individually obtained to show degradation effectiveness.

Quantification of released amino acids by UHPLC-MS/MS

Sample Preparation

Supernatant samples of approximately 8 ml of each obtained by the above 4 methods were sent in triplicate on dry ice to Shanghai Profleader Biotech Co., Ltd (Shanghai, China). Upon arrival, 100 µl of each sample was immediately added to 400 µl of 80%

415 methanol (Shanghai Ampere Laboratory Technology Co., Ltd., Shanghai, China) and
416 allowed to stand for 30 min at 40 °C, followed by centrifugation at 15,000 g and 4 °C
417 for 15 min to precipitate the proteins. Afterward, the supernatant was isometrically
418 mixed with the internal standards labeled with stable isotopes (Sigma-Aldrich,
419 Shanghai, China) for UHPLC-MS/MS analysis.

420 **UHPLC-MS/MS analysis**

421 Simultaneous quantification of 20 free amino acids without any derivatization was
422 conducted on an Agilent 1290 Infinity II UHPLC system coupled to a 6470A Triple
423 Quadrupole mass spectrometry (Santa Clara, CA, United States). Samples were pumped
424 into an ACQUITY UPLC® BEH Amide column from Waters (Milford, USA) with a
425 dimension of 100 × 2.1 mm, a particle size of 1.7 μm and a porosity of 130 Å at a
426 flowrate of 0.2 mL/min. The mobile solution (A) consisted of 10 mM ammonium
427 acetate (LC-MS grade) and (B) 90% acetonitrile. The chromatographic separation was
428 carried out with a flowrate of 0.2 ml/min, a column temperature of 40°C, and a gradient
429 elution program as follows: 0-1 min = 90%, 4 min = 85%, 12 min = 70%, 24-16 min =
430 50%, and 16.1-20 min = 90%.

431 The eluted analysts were then directly ionized with an electrospray ionization source
432 in positive mode with the following operating parameters: drying gas flow (5 L/min),
433 drying gas temperature 300°C, nebulizer gas (45 psi), sheath gas flow (11 L/min),
434 sheath gas temperature (350°C), and capillary voltage (4000 V). The detection was
435 achieved with an Agilent electron multiplier in dynamic multiple reaction monitoring

(dMRM) mode. The optimized dMRM transition (precursor -> product), fragmentor voltage, and collision energy (CE) were shown in Table S1. The Agilent MassHunter software (version B.08.00) was applied for data acquisition, while the MassHunter Workstation Software (version B.08.00, default parameters) was employed for data processing under manual inspection to guarantee accurate measurements. External calibration in the range of 0.01-5 μg/mL *via* serially diluting a working solution of the standards of 20 amino acids was utilized for quantification.

Batch cultivation and growth measurement

About 5 ml of *E. coli* fresh pre-culture was inoculated into 100 ml of the appropriate medium in a 500-ml Erlenmeyer flask, and incubated at 37°C with orbital agitation of 200 rpm, the highest speed that produced negligible foaming. For monitoring cell growth, successive samples from shake flask cultures at 12-h intervals from 12 h to 120 h were collected to measure OD600 in cuvettes with a UV–2700 spectrophotometer (Shimadzu, Japan). pH was monitored offline with a pH electrode (PHS-3C, INESA Scientific Instrument Co., Ltd, Shanghai, China) if required. When measuring OD600, 3 ml of each culture was centrifuged for 2 min at 10,000 g, the supernatant was removed, and the pellet was resuspended in sterile phosphate-buffered saline (PBS), followed by serial dilutions with PBS when appropriate to remain within the range of linearity of the instrument for OD600 measurement.

Extracting 4 growth characteristics from growth data

The *grofit* R package (version 1.1.1) (48) was employed to in-depth characterize the

457 growth curves. First, model-free smoothing spline was selected for fitting the resultant
458 growth data of each replicate, considering that spline estimates growth parameters more
459 accurately than model-based fits (48). Then, the fitted growth curve was utilized to
460 estimate 4 typical growth parameters as follows: the length of lag phase (hour), the
461 maximum instantaneous growth rate represented by the maximum slope, the maximum
462 growth (OD600), and the integral (area under the curve) estimated by numerical
463 integration (49).

464 **MSP preparation and growth evaluation**

465 Around 2.524 g of KH_2PO_4, ~3.737 g of K_2HPO_4, 10 g of glucose, and 50 mg (~ 2.03
466 mM) of $MgSO_4.7H_2O$ were added into 1 L of centrifuged OSP. The resulting broth MSP
467 was then filter-sterilized by using a 0.22-µm membrane filter unit (Millex®-GP, Merck
468 Millipore Ltd. Tullagreen, Carrigtwohill, Co. Cork, Ireland). Growth experiments and
469 extraction of 4 growth parameters were conducted to show which buffered OSP
470 achieved the best performance as described above.

471 **Reference cultivation in LB medium**

472 For comparison, *E. coli* K-12 was cultivated in LB medium based on the protocol as
473 described above. Then, 4 growth parameters were extracted from the growth data of
474 each replicate by using *grofit* (version 1.1.1) (48) according to the protocol described
475 above.

476 **Viable cell enumeration**

477 Aliquots (1 ml) of the samples collected from MSP or LB cultivation at 36 h were

serially diluted 10-fold by using 9 ml of each corresponding broth. Then, 0.1 ml of adequately diluted aliquots were aseptically pipetted onto the center of LB plates in triplicate and spread uniformly by a sterile glass rod. All inoculated plates were incubated at 37°C for 24 h and the number of colony-forming units was visually enumerated. The plated dilutions were selected so that about 30-300 viable cells were spread on each plate.

Statistical analysis

Data were presented as means ± SD (standard deviations) of biological triplicate experiments. Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test with a P value of <0.05 considered to be statistically significant, while pairwise differences were performed by the Student's t-test. All statistical analyses were performed by using R.

Acknowledgments

This work was jointly supported by the Natural Science Foundation of Guangxi ([grant number 2020GXNSFAA159032]; and the Natural Science Foundation of China [grant number 32060144] for Yizhuang Zhou.

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609

610 **Figure legends**

611 Fig. 1. Developing a biolysis method based on *B. subtilis* cultivation supernatant.
612 Varying volumes of *B. subtilis* supernatant cultivated in NB and LB were compared to
613 develop the biolysis method. (A), the final overall free amino acids after 25-h
614 degradation measured by formol titration. The volume (ul) of 0.1 M NaOH required to
615 reach the endpoint titration indicates biodegradation efficiency. The growth kinetics of
616 *E. coli* K-12 in the resultant SP broths (B), and 4 growth parameters including
617 maximum growth rate (C), length of lag phase (D), maximum growth (E), and integral
618 (F). **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001, and ns, *P* >0.05, t-test
619 compared with the SP product generated by using 3 ml NB cultivation supernatant;
620 value and error bar represent mean ± standard deviation from biological triplicate

621 experiments.

622

623 Fig. 2. Comparison of 4 degradation methods in terms of degradation efficiency and
624 growth performance. (A), the degradation efficiency measured by formol titration. The
625 volume (ul) of 0.1 M NaOH required to reach the endpoint titration indicates
626 degradation efficiency. For details, please refer to Materials and methods. The growth
627 kinetics of *E. coli* K-12 in the SP broths generated by the 4 methods (B), as well as 4
628 extracted growth parameters including maximum growth rate (C), length of lag phase
629 (D), maximum growth (E), and integral (F). **P* <0.05, ****P* <0.001, *****P* <0.0001,
630 and ns, *P* >0.05, t-test compared with the biolysis method; all experiments were
631 performed in triplicate; error bar, mean ± SD.

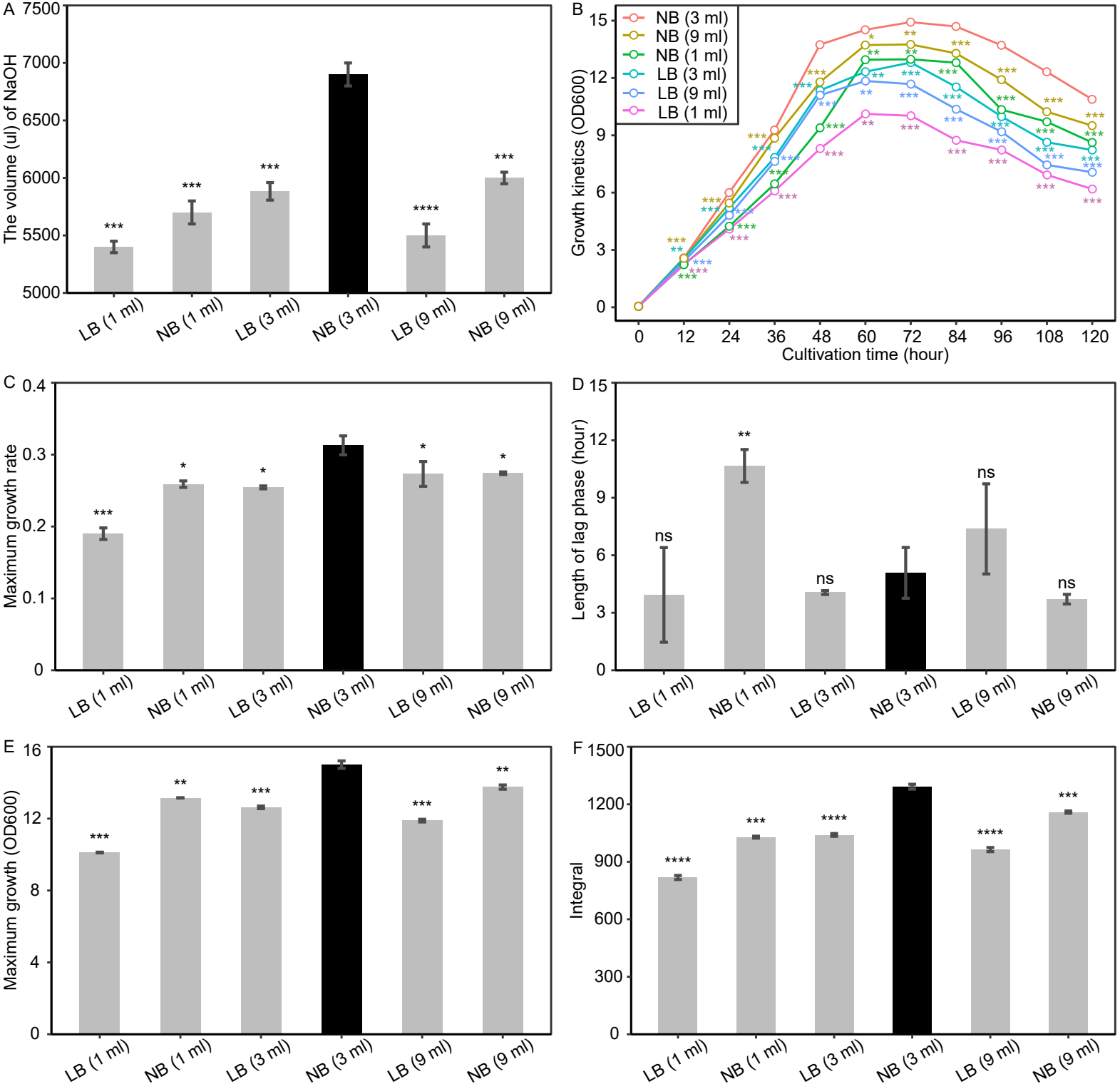
632

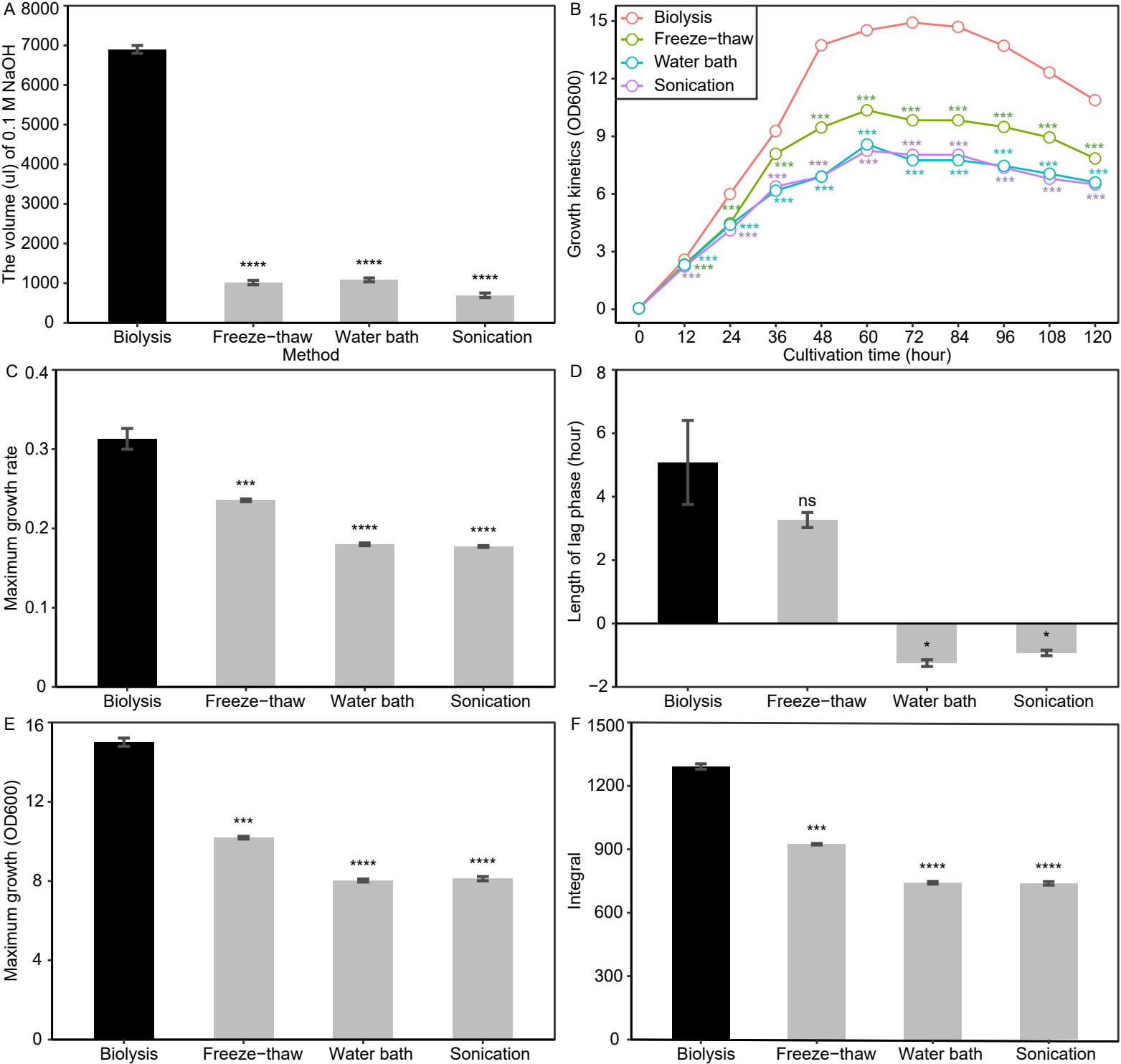
633 Fig. 3. Demonstration of the elevated degradation effectiveness by our biolysis method
634 in comparison to three mechanical methods by SDS-PAGE. SDS-PAGE profile of
635 protein degradation by the 4 methods (A), as well as the percentage of area intensity for
636 <10 kDa (B), the ranges of 10-15 kDa (C) and 15-20 kDa (D), and >20 kDa (E)
637 respectively. **P* <0.05, ***P* <0.01, two-side t-test compared with the biolysis method;
638 all experiments were performed in triplicate; error bar, mean ± SD.

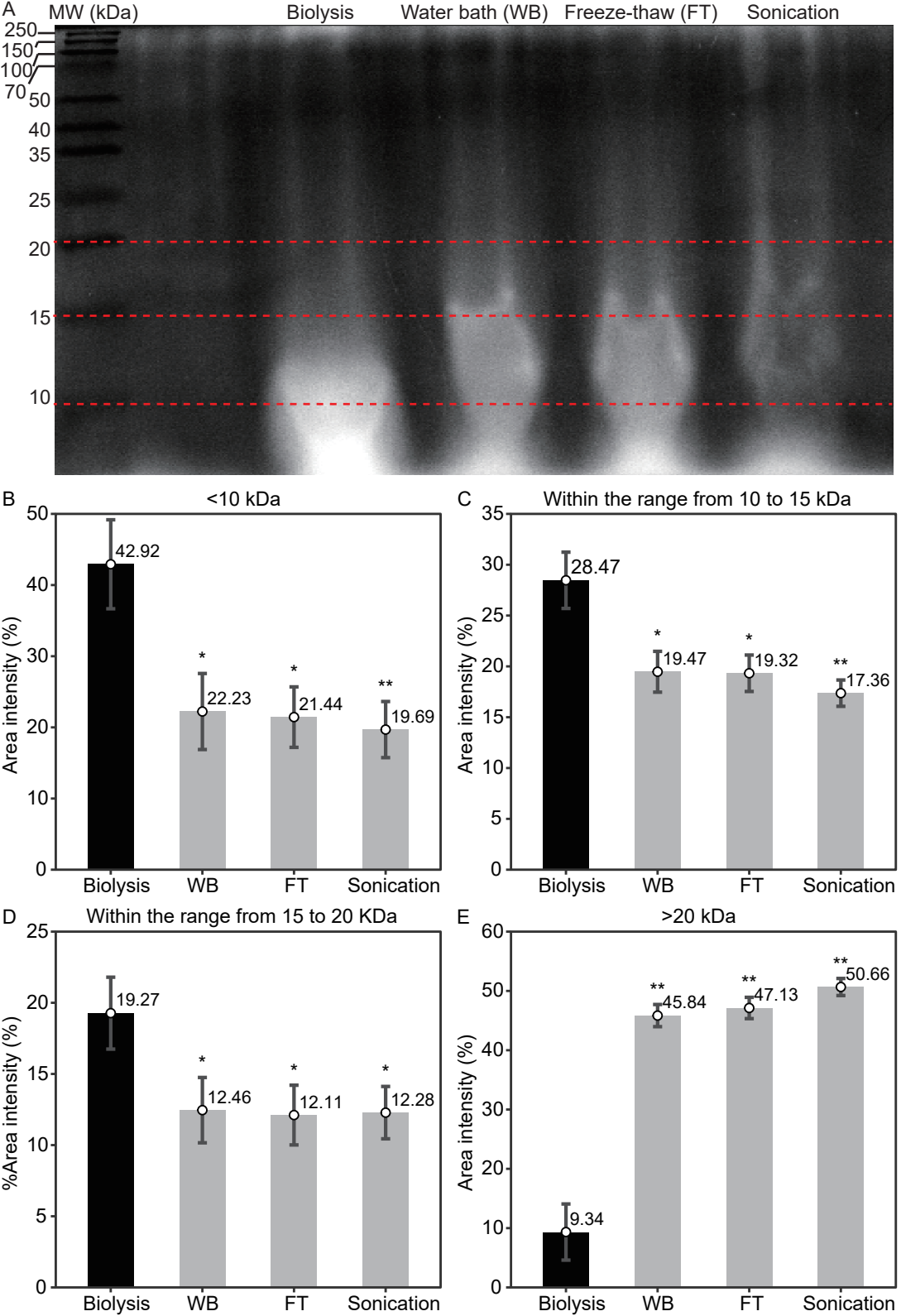
639

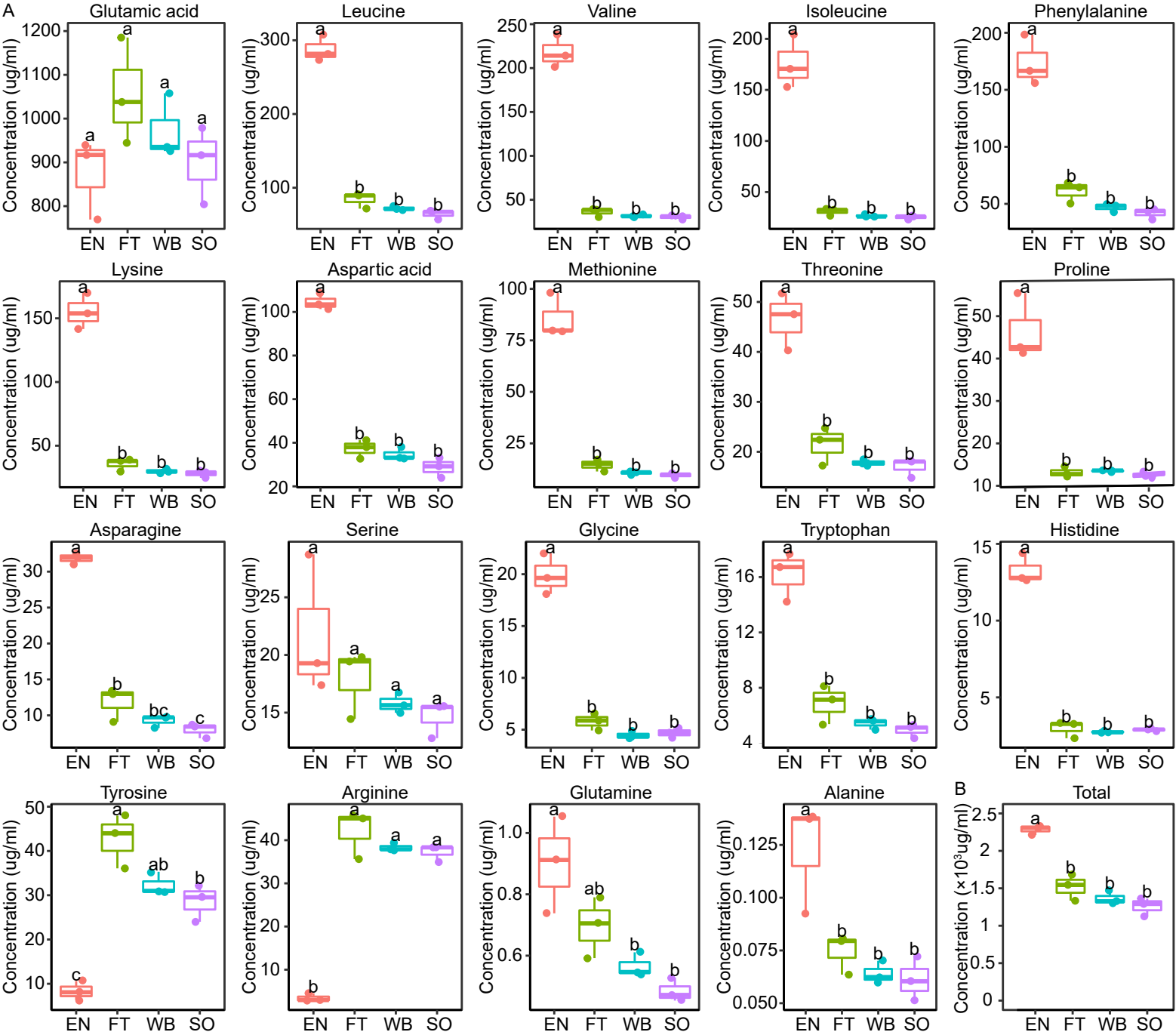
640 Fig. 4. UHPLC-MS/MS profile of individual or total amino acids liberated by the 4
641 methods. A, for 19 single amino acids; B, for total amino acids. Different low-case

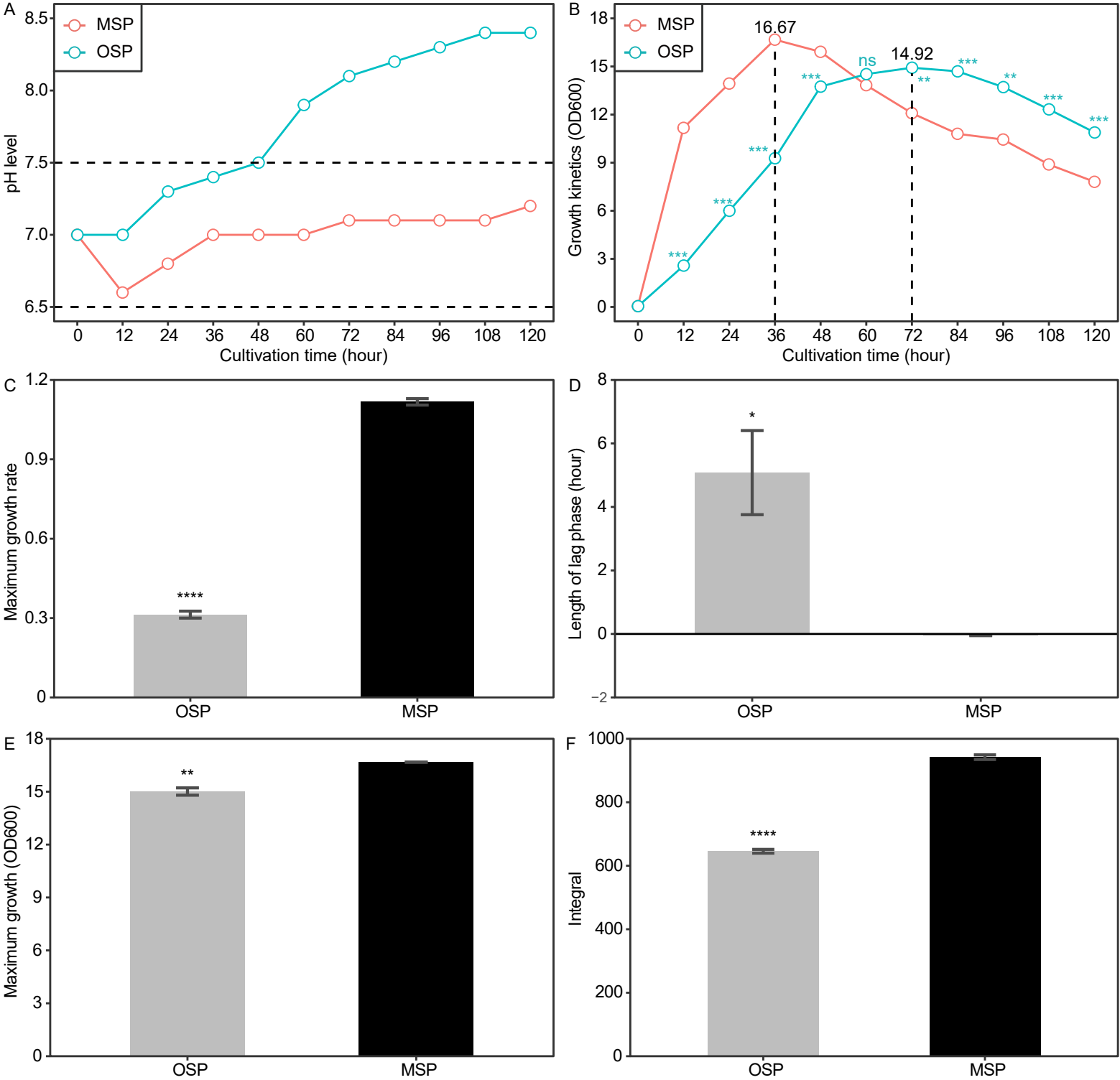
642 letters indicate statistical difference at P <0.05, one-way ANOVA followed by Tukey's
643 post hoc test (n =3 in each group); EN, the biolysis method; FT, the alternate freezing
644 and thawing; WB, the water bath method; SO, the sonication method. The total amount
645 of free amino acids was calculated by summing concentrations of all detected amino
646 acids.
647
648 Fig. 5. Improved growth performance by formulating OSP into MSP. pH maintenance
649 (A) and growth curves (B), with 4 growth parameters including maximum growth rate
650 (C), length of lag phase (D), maximum growth (E), and integral (F). Values and error
651 bars represent means ± standard deviations from biological triplicate cultivations. *P
652 <0.05, **P <0.01, ***P <0.001, ****P <0.0001, and ns, P >0.05, t-test compared with
653 MSP. The best pH range of 6.5-7.5 for *E. coli* cultivation (39), as indicated by the
654 dashed horizontal lines in panel A. The integral parameter shown here was based on the
655 cultivation until 72 h, as indicated by the dashed vertical line in panel B.
656
657 Fig. 6. Outstanding growth performance of MSP in comparison to LB. Growth curves
658 (A), maximum growth rate (B), length of lag phase (C), maximum growth (D), integral
659 (E), and the amounts of viable cells cultivated at 36 h. Values and error bars represent
660 means ± standard deviations from biological triplicate cultivations. *P <0.05, ***P
661 <0.001 and ****P <0.0001, t-test compared with MSP. Dashed vertical lines in panel A
662 indicate the times to reach the maximum OD600.
663

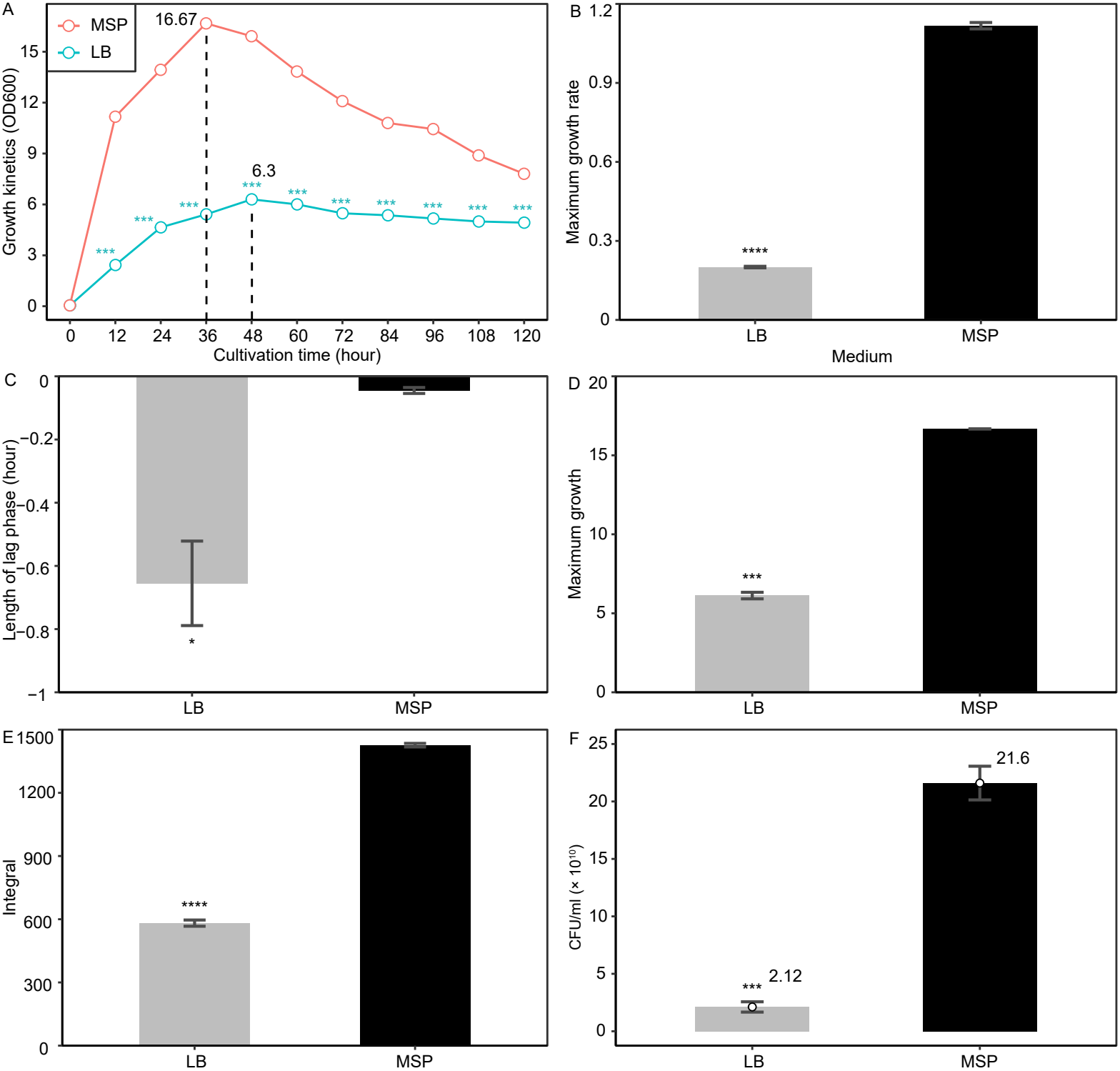

A
The volume (ul) of NaOH
7500
7000
6500
6000
5500
5000
***
***
***
****
***
LB (1 ml)
NB (1 ml)
LB (3 ml)
NB (3 ml)
LB (9 ml)
NB (9 ml)
B
Growth kinetics (OD600)
15
12
9
6
3
0
NB (3 ml)
NB (9 ml)
NB (1 ml)
LB (3 ml)
LB (9 ml)
LB (1 ml)
0
12
24
36
48
60
72
84
96
108
120
Cultivation time (hour)
C
Maximum growth rate
0.4
0.3
0.2
0.1
0
***
*
*
*
*
LB (1 ml)
NB (1 ml)
LB (3 ml)
NB (3 ml)
LB (9 ml)
NB (9 ml)
D
Length of lag phase (hour)
15
12
9
6
3
0
ns
**
ns
ns
ns
ns
LB (1 ml)
NB (1 ml)
LB (3 ml)
NB (3 ml)
LB (9 ml)
NB (9 ml)
E
Maximum growth (OD600)
16
12
8
4
0
***
**
***
***
**
LB (1 ml)
NB (1 ml)
LB (3 ml)
NB (3 ml)
LB (9 ml)
NB (9 ml)
F
Integral
1500
1200
900
600
300
0
****
***
****
****
***
LB (1 ml)
NB (1 ml)
LB (3 ml)
NB (3 ml)
LB (9 ml)
NB (9 ml)


A
The volume (ul) of 0.1 M NaOH
8000
7000
6000
5000
4000
3000
2000
1000
0
****
****
****
Biolysis
Freeze−thaw
Water bath
Sonication
Method
B
Growth kinetics (OD600)
15
12
9
6
3
0
Biolysis
Freeze−thaw
Water bath
Sonication
0
12
24
36
48
60
72
84
96
108
120
Cultivation time (hour)
C
Maximum growth rate
0.4
0.3
0.2
0.1
0
***
****
****
Biolysis
Freeze−thaw
Water bath
Sonication
D
Length of lag phase (hour)
8
6
4
2
0
−2
ns
*
*
Biolysis
Freeze−thaw
Water bath
Sonication
E
Maximum growth (OD600)
16
12
8
4
0
***
****
****
Biolysis
Freeze−thaw
Water bath
Sonication
F
Integral
1500
1200
900
600
300
0
***
****
****
Biolysis
Freeze−thaw
Water bath
Sonication


A
MW (kDa)
Biolysis
Water bath (WB)
Freeze-thaw (FT)
Sonication
250
150
100
70
50
40
35
25
20
15
10
B
<10 kDa
Area intensity (%)
42.92
22.23
21.44
19.69
C
Within the range from 10 to 15 kDa
28.47
19.47
19.32
17.36
D
Within the range from 15 to 20 KDa
%Area intensity (%)
19.27
12.46
12.11
12.28
E
>20 kDa
9.34
45.84
47.13
50.66
Biolysis
WB
FT
Sonication


A
Glutamic acid
Leucine
Valine
Isoleucine
Phenylalanine
Lysine
Aspartic acid
Methionine
Threonine
Proline
Asparagine
Serine
Glycine
Tryptophan
Histidine
Tyrosine
Arginine
Glutamine
Alanine
B
Total
Concentration (ug/ml)
Concentration (×10³ug/ml)
EN
FT
WB
SO


A
MSP
OSP
pH level
8.5
8.0
7.5
7.0
6.5
0
12
24
36
48
60
72
84
96
108
120
Cultivation time (hour)
B
MSP
OSP
16.67
14.92
ns
***
**
Growth kinetics (OD600)
18
15
12
9
6
3
0
Cultivation time (hour)
C
Maximum growth rate
1.2
0.9
0.6
0.3
0
****
OSP
MSP
D
Length of lag phase (hour)
8
6
4
2
0
−2
*
OSP
MSP
E
Maximum growth (OD600)
18
15
12
9
6
3
0
**
OSP
MSP
F
Integral
1000
800
600
400
200
0
****
OSP
MSP


A
MSP
LB
16.67
6.3
Growth kinetics (OD600)
Cultivation time (hour)
B
Maximum growth rate
Medium
LB
MSP
C
Length of lag phase (hour)
D
Maximum growth
E
Integral
F
CFU/ml (× 10^10)
21.6
2.12