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Yuan, Yuan, Chu, Depeng, Fan, Jiajun orcid.org/0000-0003-3721-5745 et al. (10 more authors) (2021) Ecofriendly conversion of algal waste into valuable plant growth-promoting rhizobacteria (PGPR) biomass. *Waste Management*. pp. 576-584. ISSN 0956-053X

<https://doi.org/10.1016/j.wasman.2020.10.020>

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Waste Management

Ecofriendly conversion of algal waste into valuable plant growth-promoting rhizobacteria (PGPR) biomass

--Manuscript Draft--

Manuscript Number:	WM-20-2146R1
Article Type:	Short Communication
Section/Category:	Biological treatment
Keywords:	Algal waste; PGPR; Microwave; Cultivation; Pepper
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Abstract:	<p>With the development of marine biorefinery concept, utilisation of algal waste during industrial processing as well as some “green tide” waste biomass has become an important research topic. In this work, a single-step microwave process was used to hydrolyse <i>Laminaria japonica</i> processing waste (LJW) and <i>Enteromorpha prolifera</i> (EP), producing a growth medium suitable for microbial cultivation. The medium contained a range of mono- and polysaccharides as well as macro- and micronutrients that could be used by the microbes. The cultivation behavior of three plant growth-promoting rhizobacteria (PGPR) strains (<i>Bacillus subtilis</i> strain Tpb55, <i>Bacillus amyloliquefaciens</i> strain Cas02, and <i>Burkholderia pyrrocinia</i> strain Lyc2) in the two media were investigated. LJW hydrolysate from 180 °C and EP hydrolysate from 150 °C performed better cultivation efficiency than those hydrolysates from other microwave conditions. Saccharide analysis showed that microbes metabolized some monosaccharide such as glucose, mannose during cultivation, leaving polysaccharide unused in the medium. Furthermore, hydrolysate-strain cultivation mixtures were applied to pepper growth. The EP hydrolysate-Cas02 broth showed better plant growth-promoting effect compared to other treatments, which might be attributed to the higher indole-3-acetic acid (IAA) production of Cas02 in the EP hydrolysate. This work shed lights on the conversion of algal waste to PGPR biomass as well as the co-application of algal hydrolysates- strains cultivation broth for a better plant growth promotion.</p>

We have carefully revised our manuscript following reviewer's comment and suggestion. Thank you very much for your time.

Reviewer Number	Reviewer Comment	Author' Response	Revised Text
#1	1.It is suggested to rewrite the introduction. Authors didn't cite from all modern references. I propose to see reference (10.1016/j.matpr.2019.08.071; https://doi.org/10.1016/j.cis.2020.102160 , https://doi.org/10.1016/j.jcis.2020.03.105 ; https://doi.org/10.1016/j.jece.2019.103075 , https://doi.org/10.1016/j.jece.2018.02.009 , https://doi.org/10.1007/s11356-020-08039-1 , https://doi.org/10.1016/j.seppur.2019.116286 , https://doi.org/10.1016/j.molliq.2020.113832).	We have made some changes to the introduction. However, the references suggested by the reviewer are about the photocatalysis of some novel materials (e.g. BiPO ₄ , Ca ₃ (PO) ₄), which are not quite relevant to our research. Therefore, we think it is not suitable to cite these references.	Page 4, line 81-84
	2. Manuscript contains some typographical errors. Make sure that these should be avoid during revision	Corrected	
	3. Conclusions section should be improved. It looks similar to the abstract. further recommendations or indicative importance or relevance to the field should be discussed	We have made corrections.	Page 15, line 351-366
#2	1. There are many English grammar mistakes in the text. A detailed check is required in the further revision.	Corrected	
	2. In the introduction section, the authors did not mention the reasons or advantages of using the microwave.	We have made some changes to the introduction.	Page 4, line 81-84
	3. The reviewer suggests the authors change Fig. 1B to a table, which will make it clearer to describe the elemental composition and C/N data.	According to Guide for Authors, articles are limited to a combined total of 8 tables and figures, therefore, we presented our results in the combined form of Fig. 1A and 1B.	

	4. A lot of abbreviations were used in this manuscript and sometimes repeated. It had better list them one by one.	We have listed abbreviations	Page 3, line 45- line 60
	5. The conclusion needs to be rewritten with more content.	We have made corrections.	Page 15, line 351- 366
#3	Some grammar and typing errors must be revised	Corrected	

Highlights

- Up to 50% (w/w%) hydrolysate yield was obtained from waste algal biomass.
- Microwave-assisted hydrolysis process is a practical approach.
- PGPR strains were successfully cultivated in hydrolysate without extra nutrients.
- Pepper growth was significantly promoted by hydrolysate-PGPR cultivation broth.

1 Ecofriendly conversion of algal waste into valuable plant growth-
2 promoting rhizobacteria (PGPR) biomass

3
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23 Abstracts

24 With the development of marine biorefinery concept, utilisation of algal waste during
25 industrial processing as well as some “green tide” waste biomass has become an
26 important research topic. In this work, a single-step microwave process was used to
27 hydrolyse *Laminaria japonica* processing waste (LJW) and *Enteromorpha prolifera* (EP),
28 producing a growth medium suitable for microbial cultivation. The medium contained
29 a range of mono- and polysaccharides as well as macro- and micronutrients that could
30 be used by the microbes. The cultivation behavior of three plant growth-promoting
31 rhizobacteria (PGPR) strains (*Bacillus subtilis* strain Tpb55, *Bacillus amyloliquefaciens*
32 strain Cas02, and *Burkholderia pyrrocinia* strain Lyc2) in the two media were
33 investigated. LJW hydrolysate from 180°C and EP hydrolysate from 150°C performed
34 better cultivation efficiency than those hydrolysates from other microwave conditions.
35 Saccharide analysis showed that microbes metabolized some monosaccharide such as
36 glucose, mannose during cultivation, leaving polysaccharide unused in the medium.
37 Furthermore, hydrolysate-strain cultivation mixtures were applied to pepper growth.
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39 compared to other treatments, which might be attributed to the higher indole-3-
40 acetic acid (IAA) production of Cas02 in the EP hydrolysate. This work shed lights on
41 the conversion of algal waste to PGPR biomass as well as the co-application of algal
42 hydrolysates- strains cultivation broth for a better plant growth promotion.

43 **Keywords:** Algal waste; PGPR; Microwave; Cultivation; Pepper

44

45 **Abbreviations**

46 PGPR -- plant growth-promoting rhizobacteria

47 LJW -- *Laminaria japonica* processing waste

48 EP -- *Enteromorpha prolifera*

49 IAA -- indole-3-acetic acid

50 LB – Luria-Bertani

51 TFA -- trifluoroacetic acid

52 NTC – None treatment control

53 Man – mannose

54 Rha – rhamnose

55 GlcA – glucuronic acid

56 GalA – galacturonic acid

57 Glc – glucose

58 Gal – galactose

59 Xyl – xylose

60 Fuc – fucose

61 1. Introduction

62 Macroalgae offer a credible feedstock for food consumption and production of
63 commodity and specialty chemicals. During the last few decades, the commercial market
64 of chemical products extracted from macroalgae has been increasing and accounting for
65 high value. The most well-established industries of these chemical products are alginate
66 from brown seaweed, agar and carrageenan from red seaweed, accounting for \$500
67 million on an annual basis([FAO, 2003](#)). Global production of wild and cultivated
68 macroalgae more than doubled from 10.4 million tonnes in 2000 to 28.4 million tonnes
69 in 2014([FAO, 2016](#)). Increased seaweed production resulted in increased seaweed waste,
70 especially from those industrial processes. For instance, carrageenan and alginate
71 production yields are generally in the range of 30-40%, leaving approximately 60-70%
72 solid waste ([Kim et al., 2013](#); [Uju et al., 2015](#)). The seaweed residue are presumed to
73 contain high concentrations of carbohydrate, which have great potential for valorisation.
74 In addition, large scale green algal blooms occurred across the world in recent years,
75 which severely endangered the costal ecology. With the development of marine
76 biorefinery concept, efficient conversion and utilisation of these waste biomass has
77 certainly become an important research topic([Yuan et al., 2018](#)).

78 Currently, macroalgae waste valorisation can be collaterally achieved by extraction of
79 bioactive compounds and conversion of biomass into renewable bioenergy via a variety
80 of thermochemical and biochemical processing methods ([Sankaran et al., 2020](#); [Yuan &](#)
81 [Macquarrie, 2015c](#)). With selective and efficient heating, microwave technology has
82 been proved to be a promising approach for treatment of biomass([Zhou et al., 2018](#)).
83 The successful demonstration of microwave reactor at both pilot and industrial scale
84 makes its real applications commercially feasible([Abeln et al., 2019](#)). Furthermore, there
85 is a huge potential for converting the macroalgae biomass through microbial processing
86 due to its high carbohydrate, nitrogen and sulphur contents. Recent research for
87 microbial conversion of macroalgae mainly focused on biofuels such as bioethanol,

88 biobutanol, biodiesel, and biogas production ([Abeln et al., 2019](#); [Tedesco & Daniels, 2018](#);
89 [Yuan & Macquarrie, 2015a](#)).

90 Plant growth-promoting rhizobacteria (PGPR) are rhizobacteria that can benefit plants
91 by stimulating growth and suppressing disease([Kloepper et al., 1980](#)). Increased
92 incidence of abiotic stresses, i.e., overuse of chemical fertilize, drought stress, soil
93 function degradation, impacting adversely plant growth and productivity in crops are
94 being witnessed all over the world. Numerous researches demonstrated that application
95 of PGPR based biocontrol agents, have great potential to increase plant growth and
96 control plant diseases ([Islam et al., 2016](#); [Myo et al., 2019](#)), in particular under stressful
97 growing conditions([Lyu et al., 2019](#)). A range of bacteria including genera of
98 *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Enterobacter*, *Burkholderia*, *Bacillus* and
99 *Serratia* have reported as PGPR to enhance plant growth ([Durairaj et al., 2018](#);
100 [Maheshwari et al., 2015](#)).

101 With the increasing use of PGPR in practice, preparation of bacterial strains, especially
102 the efficient and low-cost fermentation broth of biocontrol bacteria, have gradually
103 attracted people's attention([Iwanicki et al., 2020](#)). Different types of microorganisms
104 have a certain preference for nutritional requirements. Although a medium can be
105 produced in the laboratory to satisfy the most efficient fermentation of most
106 microorganisms, small-scale shake flask fermentation cannot meet the production
107 needs([Embaby et al., 2018](#)). Moreover, the expensive medium in the laboratory is not
108 suitable for large-scale fermentation. Generally, in microbial fermentations,
109 fermentation medium costs more than 30% of the total cost, representing a critical
110 aspects of the total cost([Salari et al., 2019](#)). The use of waste biomass or industrial by-
111 products as energy sources for PGPR production is a way to reduce costs and provide an
112 ecological alternative for waste management.

113 We, therefore, investigated the use of algal waste hydrolysates as medium for cultivation
114 of PGPR, coupling energy-efficient microwave hydrolysis of waste biomass offers

115 additional benefits for a more economic route to PGPR production([Yuan & Macquarrie,](#)
116 [2015a](#)). Three strains (*Bacillus subtilis* Tpb55, *Bacillus amyloliquefaciens* Cas02, and
117 *Burkholderia pyrrocinia* Lyc2) isolated previously were selected for their ability to
118 promote plant growth([Han et al., 2016](#); [Wang et al., 2014](#)). To the best of our knowledge,
119 this is the first report in which low cost culture medium from algal waste was used to
120 produce PGPR biomass.

121 2. Materials and reagents

122 2.1 Materials and reagents

123 The *Enteromorpha prolifera* (EP) was collected from the intertidal region from the
124 coastal area of Qingdao (36°10'N; 120°47'E), China, during August 2019. The fresh
125 seaweed was washed, air dried and ground to powder, and then stored at room
126 temperature. The *Laminaria japonica* processing waste (LJW) was the residues after
127 alginate production, and was kindly supplied by Bright Moon Seaweed Group, Qingdao,
128 China. The waste was air dried, ground and stored at room temperature. Chemicals and
129 reagents were purchased from Sigma-Aldrich for standards analytical grade.

130 2.2 Microwave assisted hydrolysis of algal waste

131 Microwave assisted hydrolysis of algal waste was carried out using a CEM Mars 6
132 microwave reactor (CEM Corporation, USA). Briefly, 2 g of dried biomass was subjected
133 to 20 mL distilled water in a 75 mL reaction tube. The sample was subsequently placed
134 in the microwave and irradiated under the dynamic mode to enable the system to
135 achieve the desired temperature (120-180 °C), and hold for 20 min. After irradiation, the
136 suspensions were centrifuged to separate the residual biomass, which was washed with
137 distilled water and dried at 105°C until constant weight. The liquid was freeze dried for
138 further use.

139 **2.3 Microorganisms**

140 *Bacillus subtilis* strain Tpb55 (CGMCC No.2853), *Bacillus amyloliquefaciens* strain Cas02
141 (CGMCC No.15514), *Burkholderia pyrrocinia* strain Lyc2 were isolated from the healthy
142 tobacco rhizosphere, Shandong province, China. All strains were preserved at -80 °C in
143 Luria-Bertani (LB) broth with 20% (v/v %) glycerol.

144 **2.4 PGPR cultivation in the algal waste hydrolysates**

145 The bacterial strains were cultured at 28°C on Luria-Bertani (LB) agar medium. To
146 prepare the seed culture of the strains, single colony of the bacteria was picked up with
147 sterilized bamboo sticks and cultured in LB broth in an orbital shaker at 28 °C and 175
148 rpm for 24 h. This pre-culture was adjusted to an optical density OD₆₀₀ of 0.3 with LB
149 medium before inoculation on algal hydrolysates. In shake flasks, 5 g/L algal hydrolysates
150 were inoculated with pre-culture at 0.1% (v/v) of total culture volume. The flasks were
151 sealed with gas-permeable film to avoid evaporation and were incubated at 28 °C and
152 175 rpm for 120 h, with readings of OD₆₀₀ performed every 24h. The OD₆₀₀ values of non-
153 inoculated algal hydrolysates were subtracted from the final OD₆₀₀. The number of viable
154 cells was determined by counting colonies formed on the LB agar plates using the plate
155 dilution method every 24 h. After cultivation, the broth cultures were centrifuged at
156 7500g for 5min and pass through a sterile microfiltration membrane (0.22-µm pore size)
157 to collect supernatant for further saccharide utilization analysis.

158 **2.5 Seeding growth test**

159 To test the application of the PGPR cultivation mixture on the growth of pepper seedlings,
160 12 treatments were designed, including EP hydrolysate-PGPR cultivation mixtures (EP+T,
161 EP+C, EP+L), LJW hydrolysate-PGPR cultivation mixtures (LJW+T, LJW+C, LJW+L), PGPR
162 suspensions (T, C, L), algal hydrolysates (EP, LJW) and water control. Briefly, bacteria were
163 cultivated in algal hydrolysates as described above for 96 h. Bacterial cells were collected

164 via centrifugation at 5000 g for 5 min at room temperature, and pellet was resuspended
165 in cultivation supernatant (for EP hydrolysate- PGPR cultivation mixtures and LJW
166 hydrolysate-PGPR cultivation mixtures) or sterile water (for PGPR suspensions) to a final
167 OD₆₀₀ of 0.3. Algal hydrolysates treatment broths were prepared by dissolving the
168 hydrolysates at 5 g/L without bacterial cultivation.

169 For the greenhouse experiment, 180 g of the sterilized soil was placed in a 10 cm
170 diameter plastic pot, and one pepper seedling with 4-5 leaves was transplanted from the
171 seedling tray to the pot. Approximately 300 pots of seedlings were prepared and
172 acclimated for one week with watering on alternate days. 18 pots were picked randomly
173 as one treatment group. Each pot received 20 mL broth of different treatments for 3
174 times at 1 week intervals. After harvest, the stem height, number of leaves, maximum
175 leaf length, maximum leaf width and dry weight of the plants were measured.

176 **2.7 Analytical methods**

177 Saccharide analysis was carried out using our previous method([Yuan et al., 2019](#)). For
178 polysaccharide compositional analysis, the samples were treated with 2M trifluoroacetic
179 acid (TFA) for 2 h at 121 °C, while liquid extracts were first nitrogen-dried before being
180 treated with TFA. The resulting monosaccharides were treated with the PMP derivation
181 method and analysed by HPLC (e2695, Waters) on a Hypersil ODS-2(C18) column with
182 UV detection. For monosaccharide analysis, the sample was directly treated with the
183 PMP derivation method and detected. The monosaccharides were quantified using
184 external calibration with an equimolar mixture of nine monosaccharide standards
185 (mannose, rhamnose, glucose, galactose, xylose, arabinose, fucose, galacturonic acid
186 and glucuronic acid).

187 Elemental analysis of carbon, hydrogen, nitrogen and sulfur contents was performed
188 using a FlashSmart Elemental Analyzer (Thermal Scientific, USA). The mineral element
189 concentrations were determined using an Atomic Absorption Spectrometer 900T

190 (PerkinElmer, USA).

191 Indole acetic acid (IAA) production was evaluated as described by ([Bric et al., 1991](#)) with
192 slight modifications. Briefly, 100 mL of algal hydrolysates medium (5 g/L) containing 5
193 mM L-tryptophan was inoculated with 100 μ L bacterial suspension (OD=0.3) and
194 incubated at 28 °C for 96 h in a shaking incubator at 175 rpm. Fully grown cultures were
195 extracted by centrifugation (8000 g; 5 min), and the supernatant (2 mL) was mixed with
196 4 mL of the Salkowski reagent (1:30:50 ratio of 0.5 M FeCl₃/ 98% (w/w) H₂SO₄/ distilled
197 water). The pink color developed was measured at 530 nm. The medium without
198 inoculation of strains was used as the control.

199 **2.8 Statistical analysis**

200 Results were presented as means \pm standard deviation. Statistical analyses were
201 determined at P <0.05 by one-way ANOVA followed by a Duncan's significant test using
202 SPSS v19.0 (SPSS Inc., Chicago, USA).

203 **3. Results and discussion**

204 **3.1 Microwave assisted hydrolysis of algal waste**

205 Microwave heating is a volumetric heat source, which can rapidly heat up water to highly
206 elevated temperatures, allowing for simple and rapid hydrolysis. Depolymerization by
207 microwave heating has been employed for a variety of lignocellulosic materials ([Fan et](#)
208 [al., 2013](#); [Mihiretu et al., 2017](#)). In this work, a single-step microwave assisted hydrolysis
209 process was applied for decomposition of algal biomass without catalytic addition.
210 Figure 1A shows the effect of temperature on the hydrolysis process. The yields of
211 hydrolysates from two waste biomass both increased with temperature, and the highest
212 yields for LJW and EP were 15.88% and 54.70% (w/w), respectively. Microwave assisted
213 hydrolysis was found to be highly suitable for *Enteromorpha prolifera*, and the

214 hydrolysates yield (>50%) was considerably higher than lignocellulosic biomass such as
215 bamboo(~20% w/w) under similar treatment conditions([Luo et al., 2017](#)). The lower
216 yield of LJW was presumably due to the pre-extraction of alginate, which removes the
217 majority of soluble materials in the *Laminaria japonica*. Additionally, the pH value of the
218 culture medium is a crucial parameter for microbial cultivation. As the temperature
219 increased, the pH value of LJW hydrolysates remained at around 8.0. This alkaline result
220 was attributed to the dilute alkaline pre-extraction of alginate. Meanwhile, the pH value
221 of EP hydrolysates significantly decreased from 7.42 at 120 °C to 5.34 at 180 °C,
222 suggesting the secondary degradation of saccharide to organic acids under severe
223 microwave conditions([Mihiretu et al., 2017](#)).

224 The elemental composition of waste biomass and hydrolysates was investigated and
225 shown in Figure 1B. Macro- and micronutrients were all abundant in hydrolysates from
226 both waste biomass, indicating their suitability for microbial processes. It is noted that
227 C/N ratio of the LJW waste biomass was 11.21, which was much lower than the
228 previously reported data of *Laminaria japonica* (28.6)([Xia et al., 2016](#)). The low ratio was
229 caused by the pre-extraction of carbohydrate content-alginate. Thus, during microwave
230 treatment, limited soluble carbohydrate was hydrolyzed, resulting in even lower C/N
231 ratios of hydrolysates (around 7). The carbon-nitrogen (C/N) ratio of *Enteromorpha*
232 *prolifera* was 26.0, in the range of reported data of macroalgae ([Korzen et al., 2015](#)).
233 Hydrolysates from EP had similar ratios with original biomass, which were 23.4 to 28.1.
234 No correlation could be elucidated between the extent of decomposition and the
235 elemental composition of the hydrolysates from waste algal biomass.

236 **3.2 Evaluation of algal waste hydrolysates as medium for PGPR cultivation**

237 Algal waste hydrolysates were directly supplied to three strains as carbon and nutrient
238 sources for cell cultivation. The growth of bacteria in hydrolysates was assessed by
239 reading OD values and counting viable cells. The results showed that all three strains
240 could grow in the hydrolysates of both algal waste without any extra nutrient added, but

241 showed different growth patterns. *B. subtilis* strain Tpb55 showed a higher OD value in
242 the LJW hydrolysates than in the EP hydrolysates, and the most suitable culture medium
243 was the LJW hydrolysate from microwave treatment at 180 °C, with an OD value of 0.45
244 for 120 h cultivation (Figure 2A). While *B. amyloliquefaciens* strain Cas02 preferred EP
245 hydrolysates, especially the one from 150 °C microwave treatment, showing an OD value
246 of 0.23 for 120 h cultivation (Figure 2B). Similar to strain Tpb55, *B. pyrrocinia* strain Lyc2
247 had the highest OD value in the LJW hydrolysate from microwave 180 °C (Figure 2C).
248 Moreover, strains Tpb55 and Cas02 were not able to grow in the slightly acidic EP
249 hydrolysate from 180 °C, while strain Lyc2 still had a growth curve in that medium,
250 indicating the better acid tolerance of Lyc2. It was also observed that the cell growth of
251 all three strains was achievable in 24 h in LB medium, whereas 24-48 h was required for
252 strains to thrive in the hydrolysates medium. This phenomenon is consistent with
253 previous studies in which bacterial strain was cultivated in acidic hydrolysates from
254 potato peel waste ([Abdelraof et al., 2019](#)). Out of expectation, the maximal OD values of
255 three strains in their favorable hydrolysates medium were all comparable with those in
256 LB medium, which could be considered attractive for future commercialization.

257 The numbers of viable cells were determined daily in these cultures. The results for 96 h
258 incubation were shown in Table S1, as the highest OD values were obtained in 96 h in
259 most cases for hydrolysates medium. The CFU ml⁻¹ values of bacteria were in good
260 agreement with the OD values. For the LJW waste hydrolysates, the CFU ml⁻¹ values were
261 similar for strains Tpb55 and Lyc2 (more than 10⁷ CFU mL⁻¹), which were higher than that
262 for strain Cas02 (less than 10⁷ CFU mL⁻¹). For the EP hydrolysates, the CFU ml⁻¹ values for
263 the three strains were in the order of Tpb55>Cas02>Lyc2. Additionally, a pH increase to
264 slightly basic value was observed in all cases (Figure 3), indicating that bacteria
265 metabolized proteins and amino acids, releasing NH₄⁺ into the medium ([Abeln et al.,
266 2019](#)).

267 The results demonstrate that microwave processing without any catalytic addition can

268 produce suitable medium from algal waste for PGPR cultivation. However, it should be
269 noted that different strains favor different medium, and selection of the best algal
270 waste/PGPR strain combination is of necessity.

271 **3.3 Carbohydrate utilization during cultivation**

272 The carbohydrate content in the substrate is considered as a key factor for suitability of
273 microbial propagation ([Xia et al., 2016](#)). According to the growth analysis of bacterial
274 strains in Section 3.2, LJW waste hydrolysate from 180 °C treatment and EP hydrolysate
275 from 150 °C were chosen as the most suitable medium for three PGPR strains. Figure 4
276 shows the carbohydrate analysis of the hydrolysates. As no catalyst was added, the
277 majority of carbohydrate were present in polysaccharide form (>95%), with less than 5%
278 in monosaccharide form in both hydrolysates. In the LJW hydrolysate, polysaccharide
279 consisted of a variety of monosaccharide, with the highest content of fucose, which is
280 the typical backbone block of fucoidan ([Yuan & Macquarrie, 2015b](#)). Thus, the major
281 polysaccharide in the LJW hydrolysate was considered to be fucoidan, a unique
282 polysaccharide found in brown macroalgae. In the EP hydrolysate, polysaccharide was
283 mainly composed of rhamnose, glucose and xylose, which is in agreement with the
284 polysaccharide extracted from green macroalgae *Enteromorpha prolifera* ([Yu et al., 2017](#)).

285 Figure 5 shows the carbohydrate content changes in the medium before and after the
286 cultivation process. All three strains consumed glucose and mannose in both
287 hydrolysates, leaving fucose in the LJW hydrolysate and rhamnose in the EP hydrolysate
288 unused, respectively (Figure 5A and 5B). This is similar to many microbial strains used for
289 biofuel fermentation such as *Saccharomyces cerevisiae*, and *Metschnikowia pulcherrima*,
290 which favor 6-carbon sugar rather than 5-carbon sugar or sugar acid ([Abeln et al., 2019](#);
291 [Yuan & Macquarrie, 2015a](#)). Furthermore, polysaccharides in both hydrolysates
292 remained unconsumed (Figure 5C and 5D), and the unchanged molecular weight of
293 polysaccharides also suggested the same results (data not shown). Recently, macroalgae
294 polysaccharides have been found to exhibit great potential to enhance plant growth, and

295 show ability to activate multiple plant defense mechanisms against a broad spectrum of
296 plant pathogens([Stadnik & de Freitas, 2014](#); [Zheng et al., 2020](#)). Therefore, it is likely that
297 the co-application of PGPR and polysaccharide in the medium after cultivation will
298 provide a stacking effect on plant growth and protection, as well as remove the cost for
299 bacterial cell separation in the overall process.

300 **3.4 Effect of hydrolysates-PGPR cultivation mixture on pepper growth**

301 To investigate whether the hydrolysates-PGPR cultivation mixture can be jointly applied
302 for promoting plant growth, 12 treatments were conducted on pepper seedlings.
303 Agricultural parameters including stem height, leaf number, maximum leaf length,
304 maximum leaf width, and dry weight of biomass were measured (Table 1). The images
305 of plants after treatment are shown in Figure 6. As can be seen, application of three
306 strains alone all showed promotions on pepper growth, but generally without significant
307 difference compared with the H₂O control, except for the leaf number of Tpb55, and
308 biomass dry weight of Tpb55 and Lyc2 (P<0.05). When applied hydrolysates-PGPR
309 cultivation mixture, the EP hydrolysate-PGPR cultivation mixtures performed much
310 better than the LJW hydrolysate-PGPR cultivation mixture, probably because of the
311 higher polysaccharide content which could promote the growth of the pepper seedlings
312 as well([El Modafar et al., 2012](#)). Although the LJW hydrolysate-PGPR cultivation mixtures
313 exhibited a positive effect on pepper growth compared with the H₂O control, there was
314 no significant difference compared with the PGPR only treatments. However, peppers
315 treated by the EP hydrolysate-PGPR cultivation mixtures not only had significantly better
316 growth than H₂O control in terms of all parameters measured (P<0.05), but also showed
317 an increased growth trend compared with PGPR only treatments. Recent study
318 demonstrated that plant polysaccharides (e.g. pectin, xylan, etc.) can serve as a carbon
319 source used to produce the extracellular matrix and can act as an environmental cue
320 that triggers biofilm formation ([Beauregard et al., 2013](#)), thereby, stimulating the
321 colonization of PGPR, which is one of key factors determining the performance of the

322 bacterium ([Xu et al., 2017](#)). This is probably the reason that the EP hydrolysate-PGPR
323 cultivation mixtures that contained a high content of polysaccharide showed better plant
324 growth-promoting effects.

325 In particular, the EP hydrolysate-Cas02 cultivation mixture showed the best plant
326 promoting effect among all treatments, with stem height, leaf number, maximum leaf
327 length, maximum leaf width, and dry weight being increased by 136%, 131%, 131%,
328 128%, and 219%, respectively, compared with the H₂O control. Nevertheless, the
329 promotion effect of EP hydrolysate-Cas02 treatment was significantly higher ($P<0.05$)
330 than that of Cas02 alone, EP hydrolysate alone, or H₂O control.

331 To explain the plant growth--promoting performance of different treatments, the
332 production of indole-3-acetic acid (IAA) of three strains in two hydrolysates media was
333 measured. PGPR play a crucial role in affecting the physiology of plants by altering their
334 hormonal balance([Ping & Boland, 2004](#)). IAA is an important phytohormone that plays a
335 major role in cell proliferation, leading to root and stem elongation([Puri et al., 2020](#)). It
336 can be seen in Table 2 that all three strains were able to synthesize IAA, but with different
337 production quantity in different hydrolysates media. Tpb55 could produce IAA in both
338 media, with 2.48 µg/mL in the EP hydrolysate medium and 1.07 µg/mL in the LJW
339 hydrolysate medium. Cas02 could only produce IAA in the EP hydrolysate medium, while
340 Lyc2 could only produce IAA in the LJW hydrolysate medium. It is also noted that strain
341 Cas02 in the EP hydrolysate medium produced significantly higher amounts of IAA
342 compared to other cultivation situations ($P<0.05$). Therefore, the best plant growth-
343 promoting effect observed for EP hydrolysate-Cas02 treatment may have resulted from
344 both the high production of IAA from Cas02 in EP the hydrolysate medium, as well as the
345 EP polysaccharides. However, in conjunction with the phytohormone modulation,
346 mechanisms including improved nutrient acquisition([Tang et al., 2020](#)), phytopathogen
347 suppression([Zhang et al., 2017](#)) and protection from abiotic stress([Singh & Jha, 2017](#))
348 have also been proved to be working during plant growth. Further work needs to be

349 conducted to deeply reveal the plant growth-promoting effects of the three strains
350 combined with algal hydrolysates.

351 **4. Conclusion**

352 Non-catalytic microwave assisted hydrolysis process could efficiently convert solid algal
353 waste to liquid hydrolysates, which were directly used as microbial cultivation growth
354 medium for PGPR without any extra nutrient added. Bacterial cells could be applied
355 alone or together with cultivation broth for plant growth-promoting purpose. The
356 combined use of the PGPR strains and the hydrolysates achieved an additive effect
357 compared with the PGPR strains or the algal hydrolysates alone. Notably, the
358 combination of strain Cas02 and EP hydrolysate showed the best results on both cell
359 cultivation and plant growth promotion application. The results paved a new path for
360 the efficient conversion and comprehensive utilisation of algal waste. However, the
361 mechanism of how the PGPR strains and the hydrolysates synergistically synergize is still
362 unclear, especially the synergetic effect between EP hydrolysate and strain Cas02
363 requires more in-depth research.

364 **Conflicts of interest**

365 There are no conflicts of interest to declare.

366 **Acknowledgement**

367 This work was supported by the Doctor Foundation of Shandong (ZR2018BC037), by the
368 National Science Foundation of China (31901937), by the Open Foundation of the
369 Ministry of Agriculture Key Laboratory of Seaweed Fertilizers (MAKLSF1813), by the
370 Central Public-interest Scientific Institutional Basal Research Fund (1610232019007,
371 1610232020004), by the Agricultural Science and Technology Innovation Program of
372 China (ASTIP-TRIC07).

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487

488 Tables and Figures

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490

491

Table 1. Growth indices of pepper with different treatment

492

Treatment	Stem height /mm	Leaf number	Maximum leaf length/mm	Maximum leaf width/mm	Dry weight /mg
H ₂ O control	88.80±3.01 ^d	8.40±0.27 ^e	70.13±2.18 ^d	37.20±1.24 ^d	61.64±10.99 ^c
T	104.60±5.17 ^{abcd}	10.20±0.24 ^{abc}	82.53±2.67 ^{abcd}	42.93±1.15 ^{abcd}	108.04±10.81 ^{ab}
C	100.40±3.50 ^{bcd}	9.53±0.29 ^{bcd}	72.80±1.94 ^d	37.33±0.64 ^d	90.08±11.45 ^{bc}
L	96.67±4.43 ^{cd}	9.73±0.33 ^{bcd}	76.60±3.56 ^{cd}	40.73±1.58 ^{bcd}	104.36±7.89 ^{ab}
EP hydrolysates	105.60±3.21 ^{abcd}	9.20±0.20 ^{cde}	81.73±2.72 ^{bcd}	42.40±1.14 ^{bcd}	85.08±4.75 ^{bc}
EP+T	124.00±5.67 ^a	10.60±0.24 ^{ab}	85.67±2.65 ^{abc}	44.40±1.50 ^{abc}	111.00±9.17 ^{ab}
EP+C	121.17±5.11 ^{ab}	11.00±0.29 ^a	92.13±3.92 ^a	47.67±2.17 ^a	135.04±12.50 ^a
EP+L	113.33±6.84 ^{abc}	10.93±0.27 ^a	90.73±2.52 ^{ab}	46.27±1.19 ^{ab}	109.60±4.76 ^{abc}
LJW hydrolysates	100.33±4.06 ^{bcd}	9.00±0.17 ^{de}	70.87±2.08 ^d	37.73±0.77 ^d	71.30±10.77 ^{bc}
LJW+T	88.93±4.14 ^d	9.07±0.28 ^{cde}	70.53±3.23 ^d	38.00±1.81 ^d	79.60±11.99 ^{abc}
LJW+C	116.53±5.90 ^{abc}	10.13±0.29 ^{abcd}	79.80±2.49 ^{bcd}	41.13±1.32 ^{bcd}	118.97±8.23 ^{abc}
LJW+L	106.13±5.68 ^{abcd}	9.67±0.21 ^{bcd}	77.67±2.55 ^{cd}	39.67±0.74 ^{cd}	89.27±1.76 ^{abc}

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494 Different letters in the same column indicate significant difference between different groups (P<0.05).

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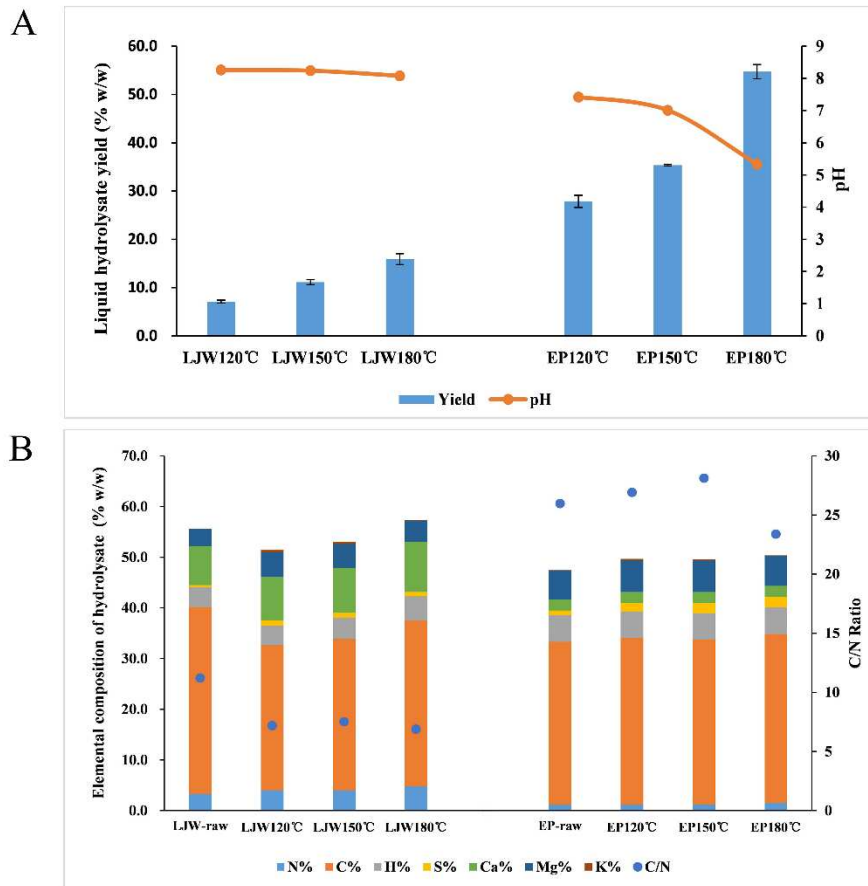
501

Table 2. Indole-3-acetic acid (IAA) production of strains in different culture media

Culture medium	Strains	IAA production (ug/mL)
EP hydrolysate	T	2.48±0.21 ^b
	C	3.74±0.23 ^a
	L	0.06±0.01 ^c
LJW hydrolysate	T	1.07±0.42 ^c
	C	0.01±0.01 ^c
	L	1.02±0.36 ^c

502 Different letters in the same column indicate significant difference between different groups (P<0.05).

503

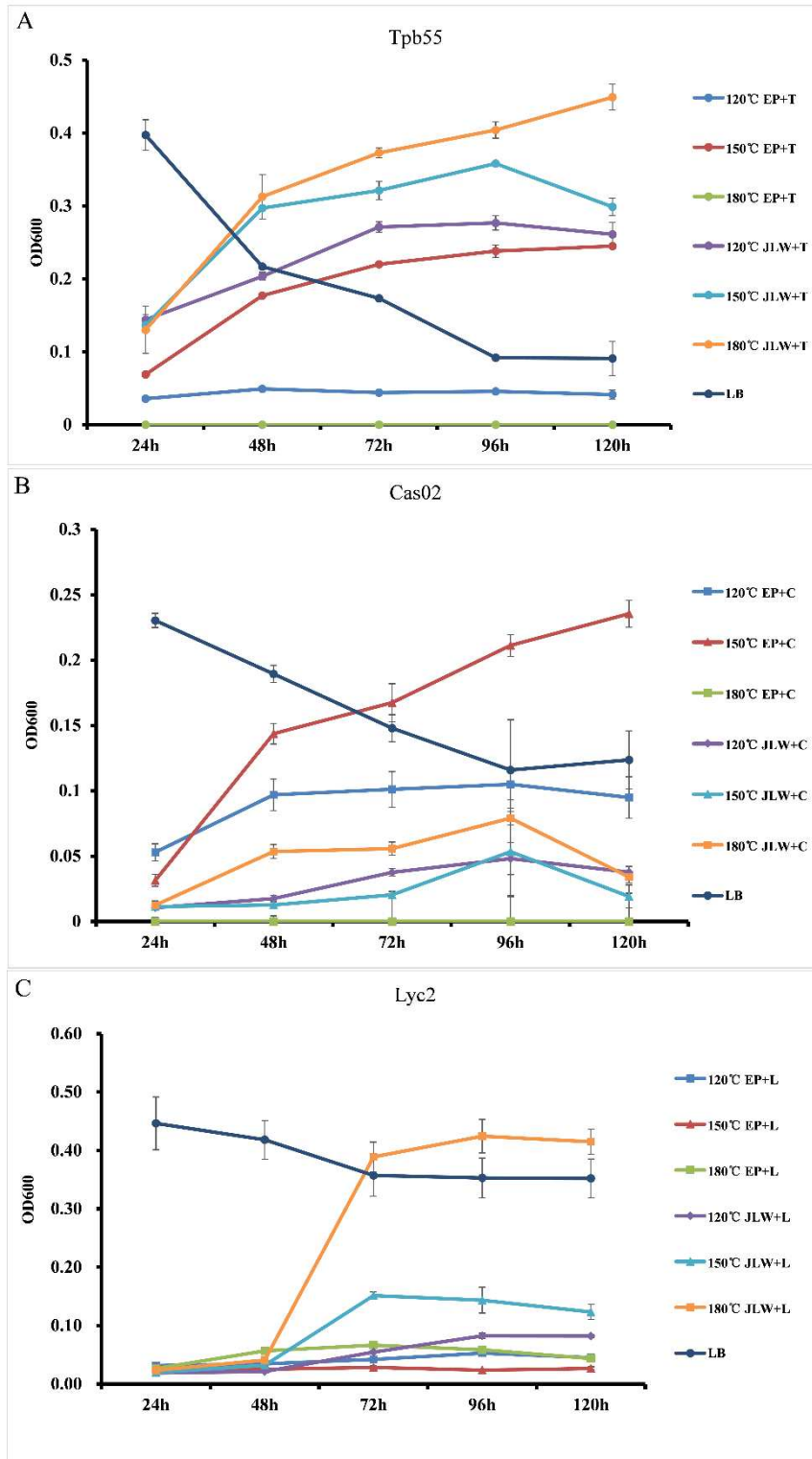


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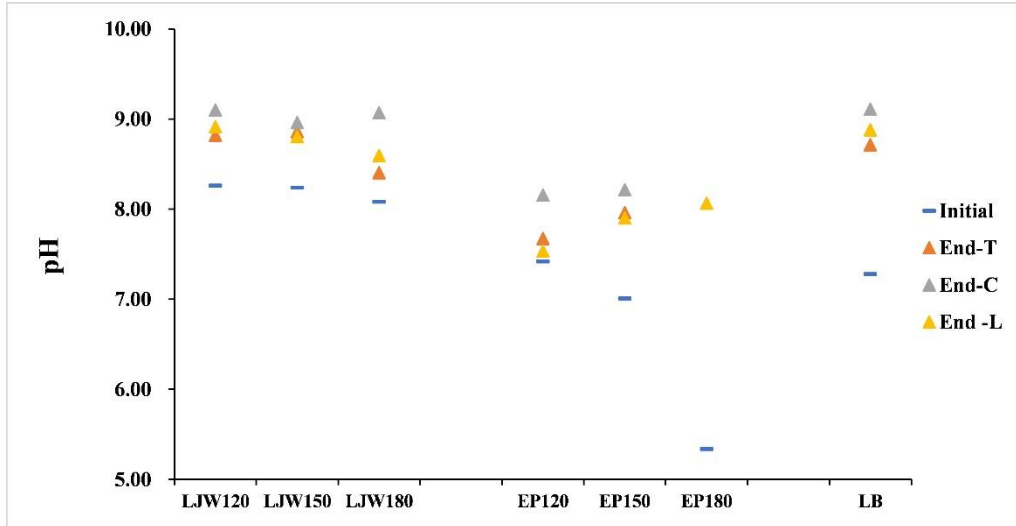
505 Figure 1. Microwave assisted hydrolysis of algae waste biomass. (A) Yields and pH of
 506 hydrolysates from different microwave conditions; (B) Elemental composition and carbo-
 507 nitrogen (C/N) ratio of hydrolysates from different microwave conditions

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510
 511 Figure 2. Growth of PGPR strains in hydrolysates from different microwave conditions.
 512 (A) *Bacillus subtilis* strain Tpb55; (B) *Bacillus amyloliquefaciens* strain Cas02; (C)
 513 *Burkholderia pyrrocinia* strain Lyc2
 514
 515



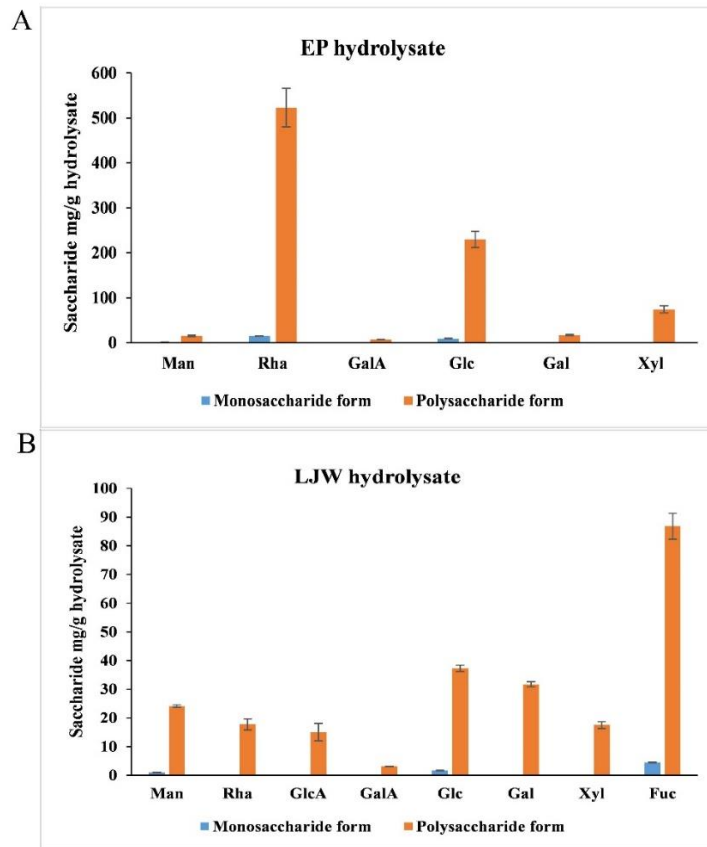
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Figure 3. pH change after 120 h cultivation of strains in different hydrolysates

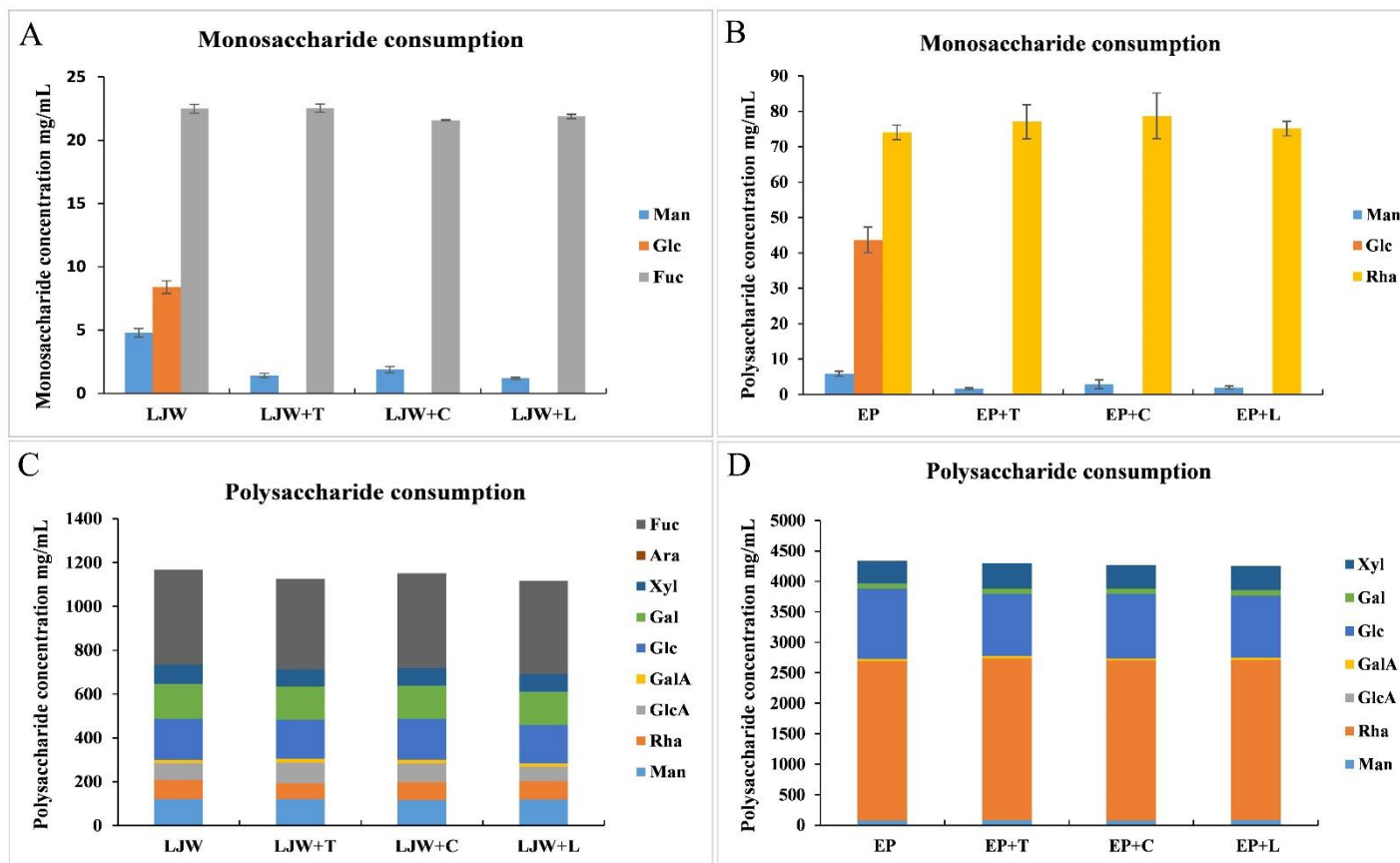


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521 Figure 4. Carbohydrate analysis of (A) EP hydrolysate from microwave assisted hydrolysis

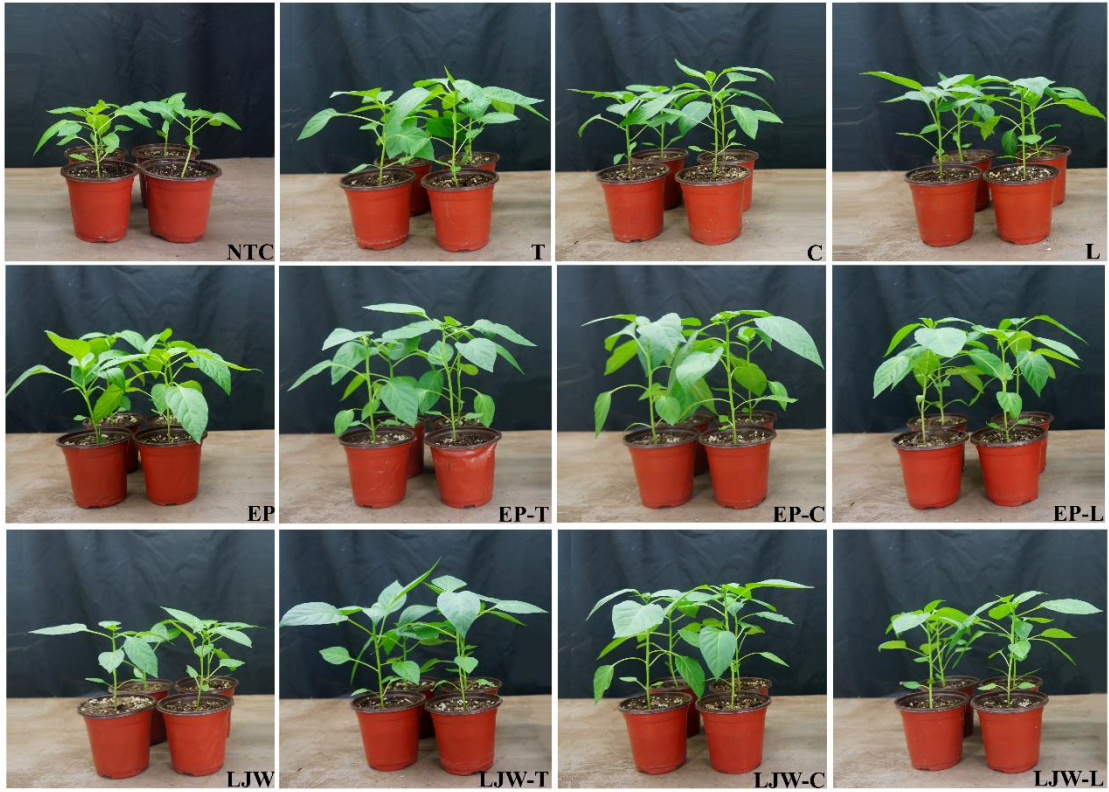
522 at 150 °C; (B) LJW hydrolysate from microwave assisted hydrolysis at 180 °C

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525 Figure 5. Carbohydrate consumption of PGPR during cultivation in hydrolysates from two algae waste biomass. Monosaccharide consumption of
 526 three strains in (A) LJW hydrolysate from microwave assisted hydrolysis at 180 °C and (B) EP hydrolysate from microwave assisted hydrolysis at
 527 150 °C; Polysaccharide consumption of three strains in (C) LJW hydrolysate from microwave assisted hydrolysis at 180 °C and (B) EP hydrolysate
 528 from microwave assisted hydrolysis at 150 °C



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Figure 6. Effect of different treatments on pepper growth

1 Ecofriendly conversion of algal waste into valuable plant growth-
2 promoting rhizobacteria (PGPR) biomass

3

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22

23 Abstracts

24 With the development of marine biorefinery concept, utilisation of algal waste during
25 industrial processing as well as some “green tide” waste biomass has become an
26 important research topic. In this work, a single-step microwave process was used to
27 hydrolyse *Laminaria japonica* processing waste (LJW) and *Enteromorpha prolifera* (EP),
28 producing a growth medium suitable for microbial cultivation. The medium contained
29 a range of mono- and polysaccharides as well as macro- and micronutrients that could
30 be used by the microbes. The cultivation behavior of three plant growth-promoting
31 rhizobacteria (PGPR) strains (*Bacillus subtilis* strain Tpb55, *Bacillus amyloliquefaciens*
32 strain Cas02, and *Burkholderia pyrrocinia* strain Lyc2) in the two media were
33 investigated. LJW hydrolysate from 180°C and EP hydrolysate from 150°C performed
34 better cultivation efficiency than those hydrolysates from other microwave conditions.
35 Saccharide analysis showed that microbes metabolized some monosaccharide such as
36 glucose, mannose during cultivation, leaving polysaccharide unused in the medium.
37 Furthermore, hydrolysate-strain cultivation mixtures were applied ~~on~~to pepper
38 growth. The EP hydrolysate-Cas02 broth showed better plant growth-promoting effect
39 compared to other treatments, which might be attributed to the higher indole-3-
40 acetic acid (IAA) production of Cas02 in the EP hydrolysate. This work ~~opens a new~~
41 avenue of shed lights on the conversion of algal waste to PGPR biomass as well as the
42 co-application of algal hydrolysates- strains cultivation broth for a better plant growth
43 promotion.

44 **Keywords:** Algal waste; PGPR; Microwave; Cultivation; Pepper

45

- 46 **Abbreviations**
- 47 PGPR -- plant growth-promoting rhizobacteria
- 48 LJW -- *Laminaria japonica* processing waste
- 49 EP -- *Enteromorpha prolifera*
- 50 IAA -- indole-3-acetic acid
- 51 LB – Luria-Bertani
- 52 TFA -- trifluoroacetic acid
- 53 NTC – None treatment control
- 54 Man – mannose
- 55 Rha – rhamnose
- 56 GlcA – glucuronic acid
- 57 GalA – galacturonic acid
- 58 Glc – glucose
- 59 Gal – galactose
- 60 Xyl – xylose
- 61 Fuc – fucose

62 1. Introduction

63 Macroalgae offer a credible feedstock for food consumption and production of
64 commodity and specialty chemicals. During the last few decades, the commercial market
65 of chemical products extracted from macroalgae has been increasing and accounting for
66 high value. The most well-established industries of these chemical products are alginate
67 from brown seaweed, ~~and~~ agar and carrageenan from red seaweed, accounting for \$500
68 million on an annual basis([FAO, 2003](#)). Global production of wild and cultivated
69 macroalgae more than doubled from 10.4 million tonnes in 2000 to 28.4 million tonnes
70 in 2014([FAO, 2016](#)). Increased seaweed production resulted in increased seaweed waste,
71 especially from those industrial processes. For instance, carrageenan and alginate
72 production yields are generally in the range of 30-40%, leaving approximately 60-70%
73 solid waste ([Kim et al., 2013](#); [Uju et al., 2015](#)). The seaweed residue are presumed to
74 contain high concentrations of carbohydrate, which have great potential for valorisation.
75 In addition, large scale green algal blooms occurred across the world in recent years,
76 which severely endangered the costal ecology. With the development of marine
77 biorefinery concept, efficient conversion and utilisation of these waste biomass has
78 certainly become an important research topic([Yuan et al., 2018](#)).

79 Currently, macroalgae waste valorisation can be collaterally achieved by extraction of
80 bioactive compounds and conversion of biomass into renewable bioenergy via a variety
81 of thermochemical and biochemical processing methods ([Sankaran et al., 2020](#); [Yuan &](#)
82 [Macquarrie, 2015c](#)). With selective and efficient heating, microwave technology has
83 been proved to be a promising approach for treatment of biomass([Zhou et al., 2018](#)).
84 The successful demonstration of microwave reactor at both pilot and industrial scale
85 makes its real applications commercially feasible([Abeln et al., 2019](#)). Furthermore,
86 there is a huge potential for converting the macroalgae biomass through microbial
87 processing due to its high carbohydrate, nitrogen and sulphur contents. Recent research
88 for microbial conversion of macroalgae mainly focused on biofuels such as bioethanol,

89 biobutanol, biodiesel, and biogas production ([Abeln et al., 2019](#); [Tedesco & Daniels, 2018](#);
90 [Yuan & Macquarrie, 2015a](#)).

91 Plant growth-promoting rhizobacteria (PGPR) are rhizobacteria that can benefit plants
92 by stimulating growth and suppressing disease([Kloepper et al., 1980](#)). Increased
93 incidence of abiotic stresses, i.e., overuse of chemical fertilize, drought stress, soil
94 function degradation, impacting adversely plant growth and productivity in crops are
95 being witnessed all over the world. Numerous researches demonstrated that application
96 of PGPR based biocontrol agents, have great potential to increase plant growth and
97 control plant diseases ([Islam et al., 2016](#); [Myo et al., 2019](#)), in particular under stressful
98 growing conditions([Lyu et al., 2019](#)). A range of bacteria including genera of
99 *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Enterobacter*, *Burkholderia*, *Bacillus* and
100 *Serratia* have reported as PGPR to enhance plant growth ([Durairaj et al., 2018](#);
101 [Maheshwari et al., 2015](#)).

102 With the increasing use of PGPR in practice, preparation of bacterial strains, especially
103 the efficient and low-cost fermentation broth of biocontrol bacteria, have gradually
104 attracted people's attention([Iwanicki et al., 2020](#)). Different types of microorganisms
105 have a certain preference for nutritional requirements. Although a medium can be
106 produced in the laboratory to satisfy the most efficient fermentation of most
107 microorganisms, small-scale shake flask fermentation cannot meet the production
108 needs([Embaby et al., 2018](#)). Moreover, the expensive medium in the laboratory is not
109 suitable for large-scale fermentation. Generally, in microbial fermentations,
110 fermentation medium costs more than 30% of the total cost, representing a critical
111 aspects of the total cost([Salari et al., 2019](#)). The use of waste biomass or industrial by-
112 products as energy sources for PGPR production is a way to reduce costs and provide an
113 ecological alternative for waste management.

114 We, therefore, investigated the use of algal waste hydrolysates as medium for cultivation
115 of PGPR, coupling energy-efficient microwave hydrolysis of waste biomass offers

116 additional benefits for a more economic route to PGPR production([Yuan & Macquarrie,](#)
117 [2015a](#)). Three strains (*Bacillus subtilis* Tpb55, *Bacillus amyloliquefaciens* Cas02, and
118 *Burkholderia pyrrocinia* Lyc2) isolated previously were selected for their ability to
119 promote plant growth([Han et al., 2016](#); [Wang et al., 2014](#)). To the best of our knowledge,
120 this is the first report in which low cost culture medium from algal waste ~~were~~was used
121 to produce PGPR biomass.

122 2. Materials and reagents

123 2.1 Materials and reagents

124 The *Enteromorpha prolifera* (EP) was collected from the intertidal region from the
125 coastal area of Qingdao (36°10'N; 120°47'E), China, during August 2019. The fresh
126 seaweed was washed, air dried and ground to powder, and then stored at room
127 temperature. The *Laminaria japonica* processing waste (LJW) was the residues after
128 alginate production, and was kindly supplied by Bright Moon Seaweed Group, Qingdao,
129 China. The waste was air dried, ground and stored at room temperature. Chemicals and
130 reagents were purchased from Sigma-Aldrich for standards analytical grade.

131 2.2 Microwave assisted hydrolysis of algal waste

132 Microwave assisted hydrolysis of algal waste was carried out using a CEM Mars 6
133 microwave reactor (CEM Corporation, USA). Briefly, 2 g of dried biomass was subjected
134 to 20 mL distilled water in a 75 mL reaction tube. The sample was subsequently placed
135 in the microwave and irradiated under the dynamic mode to enable the system to
136 achieve the desired temperature (120-180 °C), and hold for 20 min. After irradiation, the
137 suspensions were centrifuged to separate the residual biomass, which was washed with
138 distilled water and dried at 105°C until constant weight. The liquid was freeze dried for
139 further use.

140 2.3 Microorganisms

141 *Bacillus subtilis* strain Tpb55 (CGMCC No.2853), *Bacillus amyloliquefaciens* strain Cas02
142 (CGMCC No.15514), *Burkholderia pyrrocinia* strain Lyc2 were isolated from the healthy
143 tobacco rhizosphere, Shandong province, China. All strains were preserved at -80 °C in
144 Luria-Bertani (LB) broth with 20% (v/v %) glycerol.

145 2.4 PGPR cultivation in the algal waste hydrolysates

146 The bacterial strains were cultured at 28°C on Luria-Bertani (LB) agar medium. To
147 prepare the seed culture of the strains, single colony of the bacteria was picked up with
148 sterilized bamboo sticks and cultured in LB broth in an orbital shaker at 28 °C and 175
149 rpm for 24 h. This pre-culture was adjusted to an optical density OD₆₀₀ of 0.3 with LB
150 medium before inoculation on algal hydrolysates. In shake flasks, 5 g/L algal hydrolysates
151 were inoculated with pre-culture at 0.1% (v/v) of total culture volume. The flasks were
152 sealed with gas-permeable film to avoid evaporation and were incubated at 28 °C and
153 175 rpm for 120 h, with readings of OD₆₀₀ performed every 24h. The OD₆₀₀ values of non-
154 inoculated algal hydrolysates were subtracted from the final OD₆₀₀. The number of viable
155 cells was determined by counting colonies formed on the LB agar plates using the plate
156 dilution method every 24 h. After cultivation, the broth cultures were centrifuged at
157 7500g for 5min and pass through a sterile microfiltration membrane (0.22-µm pore size)
158 to collect supernatant for further saccharide utilization analysis.

159 2.5 Seeding growth test

160 ~~In order to~~ To test the application of the PGPR cultivation mixture on the growth of
161 pepper seedlings, 12 treatments were designed, including EP hydrolysate-PGPR
162 cultivation mixtures (EP+T, EP+C, EP+L), LJW hydrolysate-PGPR cultivation mixtures
163 (LJW+T, LJW+C, LJW+L), PGPR suspensions (T, C, L), algal hydrolysates (EP, LJW) and water
164 control. Briefly, bacterial ~~was~~ were cultivated in algal hydrolysates as described above

165 for 96 h. Bacterial cells were collected via ~~certification~~centrifugation at 5000 g for 5 min
166 at room temperature, and pellet was resuspended in cultivation supernatant (for EP
167 hydrolysate- PGPR cultivation mixtures and LJW hydrolysate-PGPR cultivation mixtures)
168 or sterile water (for PGPR suspensions) to a final OD₆₀₀ of 0.3. Algal hydrolysates
169 treatment broths were prepared by dissolving the hydrolysates at 5 g/L without bacterial
170 cultivation.

171 For the greenhouse experiment, 180 g of the sterilized soil was placed in a 10 cm
172 diameter plastic pot, and one pepper seedling with 4-5 leaves ~~were~~was transferred
173 transplanted from the seedling tray to the pot. ~~Around~~Approximately 300 pots of
174 seedlings were prepared and acclimated for one week with watering on alternate days.
175 18 pots were picked randomly as one treatment group. Each pot received 20 mL broth
176 of different treatments for 3 times at 1 week intervals. After harvest, the stem height,
177 number of leaves, maximuma leaf length, maximumal leaf width and dry weight of the
178 plants were measured.

179 **2.7 Analytical methods**

180 Saccharide analysis was carried out using our previous method([Yuan et al., 2019](#)). For
181 polysaccharide compositional analysis, the samples ~~was~~were treated with 2M
182 trifluoroacetic acid (TFA) for 2 h at 121 °C, while liquid extracts were first nitrogen-dried
183 before being treated with TFA. The resulting monosaccharides were treated with the
184 PMP derivation method and analysed by HPLC (e2695, Waters) on a Hypersil ODS-2(C18)
185 column with UV detection. For monosaccharide analysis, the sample was directly treated
186 with the PMP derivation method and detected. The monosaccharides were quantified
187 using external calibration with an equimolar mixture of nine monosaccharide standards
188 (mannose, rhamnose, glucose, galactose, xylose, arabinose, fucose, galacturonic acid
189 and glucuronic acid).

190 Elemental analysis of carbon, hydrogen, nitrogen and sulfur contents was performed

191 using a FlashSmart Elemental Analyzer (Thermal Scientific, USA). The mineral element
192 concentrations were determined using an Atomic Absorption Spectrometer 900T
193 (PerkinElmer, USA).

194 Indole acetic acid (IAA) production was evaluated as described by ([Bric et al., 1991](#)) with
195 slight modifications. Briefly, 100 mL of algal hydrolysates medium (5 g/L) containing 5
196 mM L-tryptophan was inoculated with 100 μ L bacterial suspension (OD=0.3) and
197 incubated at 28 °C for 96 h in a shaking incubator at 175 rpm. Fully grown cultures were
198 extracted by centrifugation (8000 g; 5 min), and the supernatant (2 mL) was mixed with
199 4 mL of the Salkowski reagent (1:30:50 ratio of 0.5 M FeCl₃/ 98% (w/w) H₂SO₄/ distilled
200 water). The pink color developed was measured at 530 nm. The medium without
201 inoculation of strains was used as the control.

202 **2.8 Statistical analysis**

203 Results were presented as means \pm standard deviation. Statistical analyses were
204 determined at P <0.05 by one-way ANOVA followed by a Duncan's significant test using
205 SPSS v19.0 (SPSS Inc., Chicago, USA).

206 **3. Results and discussion**

207 **3.1 Microwave assisted hydrolysis of algal waste**

208 Microwave heating is a volumetrically heat source, which can rapidly heat up water to
209 highly elevated temperatures, allowing for simple and rapid hydrolysis.
210 Depolymerization by microwave heating has been employed for a variety of
211 lignocellulosic materials ([Fan et al., 2013](#); [Mihiretu et al., 2017](#)). In this work, a single-
212 step microwave assisted hydrolysis process was applied for decomposition of algal
213 biomass without catalytic addition. Figure 1A shows the effect of temperature on the
214 hydrolysis process. The yields of hydrolysates from two waste biomass both increased

215 with temperature, and the highest yields for LJW and EP were 15.88% and 54.70% (w/w),
216 respectively. Microwave assisted hydrolysis was found to be highly suitable for
217 *Enteromorpha prolifera*, and the hydrolysates yield (>50%) ~~is-was~~ considerably higher
218 than lignocellulosic biomass such as bamboo (~20% w/w) under similar treatment
219 conditions ([Luo et al., 2017](#)). The lower yield of LJW ~~is-was~~ presumably due to the pre-
220 extraction of alginate, which removes the majority of soluble materials in the *Laminaria*
221 *japonica*. Additionally, ~~the~~ pH value of ~~the~~ culture medium is a crucial parameter for
222 microbial cultivation. As the temperature increased, the pH value of LJW hydrolysates
223 remained at around 8.0, ~~and this~~ ~~This~~ alkaline result ~~are-was~~ attributed to the dilute
224 alkaline pre-extraction of alginate. Meanwhile, the pH value of EP hydrolysates
225 significantly decreased from 7.42 at 120 °C to 5.34 at 180 °C, suggesting the secondary
226 degradation of saccharide to organic acids under severe microwave conditions ([Mihiretu](#)
227 [et al., 2017](#)).

228 The elemental composition of waste biomass and hydrolysates ~~were-was~~ investigated
229 and shown in Figure 1B. Macro- and micronutrients were all abundant in hydrolysates
230 from both waste biomass, indicating the ~~ir~~ suitability for microbial processes. It is noted
231 that C/N ratio of the LJW waste biomass was 11.21, which was much lower than the
232 previously reported data of *Laminaria japonica* (28.6) ([Xia et al., 2016](#)). ~~This~~ ~~The~~ low ratio
233 was caused by the pre-extraction of carbohydrate content-alginate. Thus, during
234 microwave treatment, limited soluble carbohydrate was hydrolyzed, resulting in even
235 lower C/N ratios of hydrolysates (around 7). The carbon-nitrogen (C/N) ratio of
236 *Enteromorpha prolifera* was 26.0, in the range of reported data of macroalgae ([Korzen](#)
237 [et al., 2015](#)). Hydrolysates from EP had similar ratios with original biomass, which were
238 23.4 to 28.1. No correlation could be elucidated between the extent of decomposition
239 and the elemental composition of the hydrolysates from waste algal biomass.

240 3.2 Evaluation of algal waste hydrolysates as medium for PGPR cultivation

241 Algal waste hydrolysates were directly supplied to three strains as carbon and nutrient

242 sources for cell cultivation. The growth of bacteria~~l~~ in hydrolysates was assessed by
243 reading OD values and counting viable cells. The results showed that all three strains
244 could grow in the hydrolysates of both algal waste without any extra nutrient added, but
245 show~~ed~~ different growth patterns. *B. subtilis* strain Tpb55 showed a higher OD value in
246 the LJW hydrolysates than in the EP hydrolysates, and the most suitable culture medium
247 was the LJW hydrolysate from microwave treatment at 180 °C, with ~~the-an~~ OD value of
248 0.45 for 120 h cultivation (Figure 2A). While *B. amyloliquefaciens* strain Cas02 preferred
249 EP hydrolysates, especially the one from 150 °C microwave treatment, showing ~~the-an~~
250 OD value of 0.23 for 120 h cultivation (Figure 2B). Similar ~~with-to~~ strain Tpb55, *B.*
251 *pyrocinia* strain Lyc2 had ~~the~~ highest OD value in the LJW hydrolysate from microwave
252 180 °C (Figure 2C). Moreover, strains Tpb55 and Cas02 were not ~~be~~ able to grow in the
253 slight~~ly~~ acidic EP hydrolysate from 180 °C, while strain Lyc2 still had a growth curve in
254 that medium, indicating the better acid tolerance of Lyc2. It ~~is-was~~ also observed that
255 the cell growth of all three strains was achievable in 24 h in LB medium, whereas 24-48
256 h was ~~needed-required~~ for strains to ~~get-used-to~~ thrive in the hydrolysates medium. This
257 phenomenon is in ~~accordance-consistent~~ with previous studies in which bacterial strain
258 was cultivated in acid~~ic~~ hydrolysates from potato peel waste ([Abdelraof et al., 2019](#)). Out
259 of expectation, the maximal OD values of three strains in their favorable hydrolysates
260 medium were all comparable with those in LB medium, which could be considered
261 attractive for future commercialization.

262 The numbers of viable cells were determined daily in these culture~~s~~. The results for 96 h
263 incubation were shown in Table S1, as the highest OD values were obtained in 96 h in
264 most cases for hydrolysates medium. ~~As can be seen, the-The~~ CFU ml⁻¹ values of bacteria~~l~~
265 were in good agreement with ~~the~~ OD values. For the LJW waste hydrolysates, the CFU
266 ml⁻¹ values were similar for strain~~s~~ Tpb55 and Lyc2 (more than 10⁷ CFU mL⁻¹), which were
267 higher than ~~that for~~ strain Cas02 (less than 10⁷ CFU mL⁻¹). For the EP hydrolysates, the
268 CFU ml⁻¹ values for the three strains were in the order of Tpb55>Cas02>Lyc2. Additionally,
269 a pH increase to slightly basic value was observed in all cases (Figure 3), indicating that

270 bacteria~~d~~ metabolized proteins and amino acids, releasing NH₄⁺ into the medium([Abeln](#)
271 [et al., 2019](#)).

272 The results demonstrate that microwave processing without any catalytic addition can
273 produc~~e~~ing suitable medium from algal waste for PGPR cultivation. However, it should
274 be noted that different strains favor different medium, and selection of the best algal
275 waste/PGPR strain combination is of necessity.

276

277 **3.3 Carbohydrate utilization during cultivation**

278 The carbohydrate content in the substrate is considered as a key factor for suitability of
279 microbial propagation([Xia et al., 2016](#)). According to the growth analysis of bacterial
280 strains in ~~section~~Section 3.2, LJW waste hydrolysate from 180 °C treatment and EP
281 hydrolysate from 150 °C were chosen as the most suitable medium for three PGPR strains.
282 Figure 4 shows the carbohydrate analysis of the ~~above~~ hydrolysates. As no catalyst was
283 added, the majority of carbohydrate were present in polysaccharide form (>95%), with
284 less than 5% in monosaccharide form in both hydrolysates. In the LJW hydrolysate,
285 polysaccharide consisted of a variety of monosaccharide, with the highest content of
286 fucose, which is the typical backbone block of fucoidan ([Yuan & Macquarrie, 2015b](#)).
287 Thus, the major polysaccharide in the LJW hydrolysate was consider to be fucoidan, a
288 unique polysaccharide ~~from~~found in brown macroalgae. In the EP hydrolysate,
289 polysaccharide was mainly ~~composed~~composed of rhamnose, glucose and xylose,
290 which is in agreement with the polysaccharide extracted from green macroalgae
291 *Enteromorpha prolifera*([Yu et al., 2017](#)).

292 Figure 5 shows the carbohydrate content changes in the medium before and after the
293 cultivation process. All three strains consumed glucose and mannose in both
294 hydrolysates, leaving fucose in the LJW hydrolysate and rhamnose in the EP hydrolysate
295 unused, respectively (Figure 5A and 5B). This is similar ~~with~~to many microbial strains

296 used for biofuel fermentation such as *Saccharomyces cerevisiae*, and *Metschnikowia*
297 *pulcherrima*, which favor 6-carbon sugar rather than 5-carbon sugar or sugar acid ([Abeln](#)
298 [et al., 2019](#); [Yuan & Macquarrie, 2015a](#)). Furthermore, polysaccharides in both
299 hydrolysates remained unconsumed (Figure 5C and 5D), and the unchanged molecular
300 weight of polysaccharides also suggested the same results (data not shown). Recently,
301 macroalgae polysaccharides have been found to exhibit great potential to enhance plant
302 growth, and show ability to activate multiple plant defense mechanisms against a broad
303 spectrum of plant pathogens([Stadnik & de Freitas, 2014](#); [Zheng et al., 2020](#)). Therefore,
304 it is likely that the co-application of PGPR and polysaccharide in the medium after
305 cultivation will provide a stacking effect on plant growth and protection, as well as
306 remove the cost for bacterial cell separation in the overall process.

307 **3.4 Effect of hydrolysates-PGPR cultivation mixture on pepper growth**

308 To investigate whether the hydrolysates-PGPR cultivation mixture can be jointly applied
309 for promoting plant growth, 12 treatments were conducted on pepper seedlings.
310 Agricultural parameters including stem height, leaf number, ~~maximal~~maximum leaf
311 length, maximum leaf width, and dry weight of biomass were measured (Table 1). The
312 images of plants s after treatment ~~were~~are shown in Figure 6. As can be seen, application
313 of three strains alone all showed promotions on pepper growth, but generally without
314 significant difference compared with the H₂O control, except for the leaf number of
315 Tpb55, and biomass dry weight of Tpb55 and Lyc2 (P<0.05). When applied hydrolysates-
316 PGPR cultivation mixture, the EP hydrolysate-PGPR cultivation mixtures performed much
317 better than the LJW hydrolysate-PGPR cultivation mixture, probably because of the
318 higher polysaccharide content which could promote the growth of the pepper seedlings
319 as well([El Modafar et al., 2012](#)). Although the LJW hydrolysate-PGPR cultivation mixtures
320 exhibited a positive effect on pepper growth compared with the H₂O control, there ~~is~~
321 was no significant difference compared with the PGPR only treatments. However,
322 peppers treated by the EP hydrolysate-PGPR cultivation mixtures not only had

323 significantly better growth than H₂O control in terms of all parameters measured
324 (P<0.05), but also showed ~~an obviously~~ increased growth trend compared with PGPR
325 only treatments. Recently study demonstrated that plant polysaccharides (e.g. pectin,
326 xylan, etc.) can serve as a carbon source used to produce the extracellular matrix and
327 ~~can~~ act as an environmental cue that triggers biofilm formation ([Beauregard et al., 2013](#)),
328 ~~therefore~~~~thereby~~, stimulating the colonization of PGPR, which is one of key factors
329 determining the performance of the bacterium ([Xu et al., 2017](#)). This is probably ~~the~~
330 ~~reason that the~~ ~~why~~ EP hydrolysate-PGPR cultivation mixtures that contained ~~a~~ high
331 content of polysaccharide showed better plant growth ~~-promoting~~~~on~~ effects.

332 In particular, ~~the~~ EP hydrolysate-Cas02 cultivation mixture showed the best plant
333 promoting effect among all treatments, ~~-of which the~~~~with~~ stem height, leaf number,
334 ~~maximal~~~~maximum~~ leaf length, ~~maximal~~~~maximum~~ leaf width, and dry weight ~~being~~
335 increased by 136%, 131%, 131%, 128%, ~~and~~ 219%, respectively, compared with ~~the~~ H₂O
336 control. Nevertheless, the promotion effect of EP hydrolysate-Cas02 treatment was
337 significantly higher (P<0.05) than ~~that of~~ Cas02 alone, EP hydrolysate alone, ~~and or~~ H₂O
338 control.

339 To explain the plant growth ~~-promotion~~~~promoting~~ performance of different treatments,
340 ~~the~~ production of indole-3-acetic acid (IAA) of three strains in two hydrolysates media
341 was measured. PGPR play a crucial role in affecting the physiology of ~~a~~ plants ~~s~~ by altering
342 their hormonal balance([Ping & Boland, 2004](#)). IAA is an important phytohormone that
343 ~~has~~~~plays~~ a major role in cell proliferation, leading to ~~root~~~~root~~ and stem elongation([Puri](#)
344 [et al., 2020](#)). It can be seen in Table 2 that all three strains were able to synthesize IAA,
345 but with different production quantity in different hydrolysates ~~medium~~~~media~~. Tpb55
346 could produce IAA in both media, with 2.48 µg/mL in ~~the~~ EP hydrolysate medium and
347 1.07 µg/mL in ~~the~~ LJW hydrolysate medium. Cas02 could only produce IAA in ~~the~~ EP
348 hydrolysate medium, while Lyc2 could only produce IAA in ~~the~~ LJW hydrolysate medium.
349 It is also noted that strain Cas02 in ~~the~~ EP hydrolysate medium produced significantly

350 higher amounts of IAA compared to other cultivation situations ($P < 0.05$). Therefore, the
351 best plant growth-promoting effect observed for EP hydrolysate-Cas02 treatment may
352 have resulted from both the high production of IAA from Cas02 in EP the hydrolysate
353 medium, as well as the EP polysaccharides. However, ~~despite of~~ in conjunction with the
354 phytohormone modulation, mechanisms including improved nutrient acquisition ([Tang
et al., 2020](#)), phytopathogen suppression ([Zhang et al., 2017](#)) and protection from abiotic
355 stress ([Singh & Jha, 2017](#)) have also been proved to be working during ~~the~~ plant growth.
356 Further work needs to be ~~done~~ conducted to deeply reveal the plant growth-promoting
357 effects of the three strains combined with algal hydrolysates.
358

359 4. Conclusion

360 ~~Non-catalytic Microwave-microwave~~ assisted hydrolysis process could efficiently convert
361 solid algal waste to liquid hydrolysates, which were directly used as microbial cultivation
362 growth medium for PGPR ~~cultivation~~ without any extra nutrient added. Bacterial cells
363 ~~can~~ could be applied alone or together with cultivation broth for plant growth-promoting
364 purpose. The combined use of the PGPR strains and the hydrolysates achieved an
365 additive effect compared with the PGPR strains or the algal hydrolysates alone. Notably,
366 the combination of strain Cas02 and EP hydrolysate showed the best results on both cell
367 cultivation and plant growth promotion application. The results paved a new path for
368 the efficient conversion and comprehensive utilisation of algal waste. Notably, the
369 ~~combination of strain Cas02 and EP hydrolysate showed the best results on both cell~~
370 ~~cultivation and plant growth promotion application, compared with other strain~~
371 ~~hydrolysate combinations. However, the mechanism of how the PGPR strains and the~~
372 hydrolysates synergistically synergize is still unclear, especially the synergetic effect
373 between EP hydrolysate and strain Cas02 requires more in-depth research. Further
374 ~~studies are required to better understand the specific mechanism involved in synergetic~~
375 ~~effect between EP hydrolysate and strain Cas02.~~

376 Conflicts of interest

377 There are no conflicts of interest to declare.

378 Acknowledgement

379 This work was supported by the Doctor Foundation of Shandong (ZR2018BC037), by the
380 National Science Foundation of China (31901937), by the Open Foundation of the
381 Ministry of Agriculture Key Laboratory of Seaweed Fertilizers (MAKLSF1813), by the
382 Central Public-interest Scientific Institutional Basal Research Fund (1610232019007,
383 1610232020004), by the Agricultural Science and Technology Innovation Program of
384 China (ASTIP-TRIC07).

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500 Tables and Figures

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502

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Table 1. Growth indices of pepper with different treatment

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Treatment	Stem height /mm	Leaf number	Maximum leaf length/mm	Maximum leaf width/mm	Dry weight /mg
H ₂ O control	88.80±3.01 ^d	8.40±0.27 ^e	70.13±2.18 ^d	37.20±1.24 ^d	61.64±10.99 ^c
T	104.60±5.17 ^{abcd}	10.20±0.24 ^{abc}	82.53±2.67 ^{abcd}	42.93±1.15 ^{abcd}	108.04±10.81 ^{ab}
C	100.40±3.50 ^{bcd}	9.53±0.29 ^{bcd}	72.80±1.94 ^d	37.33±0.64 ^d	90.08±11.45 ^{bc}
L	96.67±4.43 ^{cd}	9.73±0.33 ^{bcd}	76.60±3.56 ^{cd}	40.73±1.58 ^{bcd}	104.36±7.89 ^{ab}
EP hydrolysates	105.60±3.21 ^{abcd}	9.20±0.20 ^{cde}	81.73±2.72 ^{bcd}	42.40±1.14 ^{bcd}	85.08±4.75 ^{bc}
EP+T	124.00±5.67 ^a	10.60±0.24 ^{ab}	85.67±2.65 ^{abc}	44.40±1.50 ^{abc}	111.00±9.17 ^{ab}
EP+C	121.17±5.11 ^{ab}	11.00±0.29 ^a	92.13±3.92 ^a	47.67±2.17 ^a	135.04±12.50 ^a
EP+L	113.33±6.84 ^{abc}	10.93±0.27 ^a	90.73±2.52 ^{ab}	46.27±1.19 ^{ab}	109.60±4.76 ^{abc}
LJW hydrolysates	100.33±4.06 ^{bcd}	9.00±0.17 ^{de}	70.87±2.08 ^d	37.73±0.77 ^d	71.30±10.77 ^{bc}
LJW+T	88.93±4.14 ^d	9.07±0.28 ^{cde}	70.53±3.23 ^d	38.00±1.81 ^d	79.60±11.99 ^{abc}
LJW+C	116.53±5.90 ^{abc}	10.13±0.29 ^{abcd}	79.80±2.49 ^{bcd}	41.13±1.32 ^{bcd}	118.97±8.23 ^{abc}
LJW+L	106.13±5.68 ^{abcd}	9.67±0.21 ^{bcd}	77.67±2.55 ^{cd}	39.67±0.74 ^{cd}	89.27±1.76 ^{abc}

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506 Different letters in the same column indicate significant difference between different groups (P<0.05).

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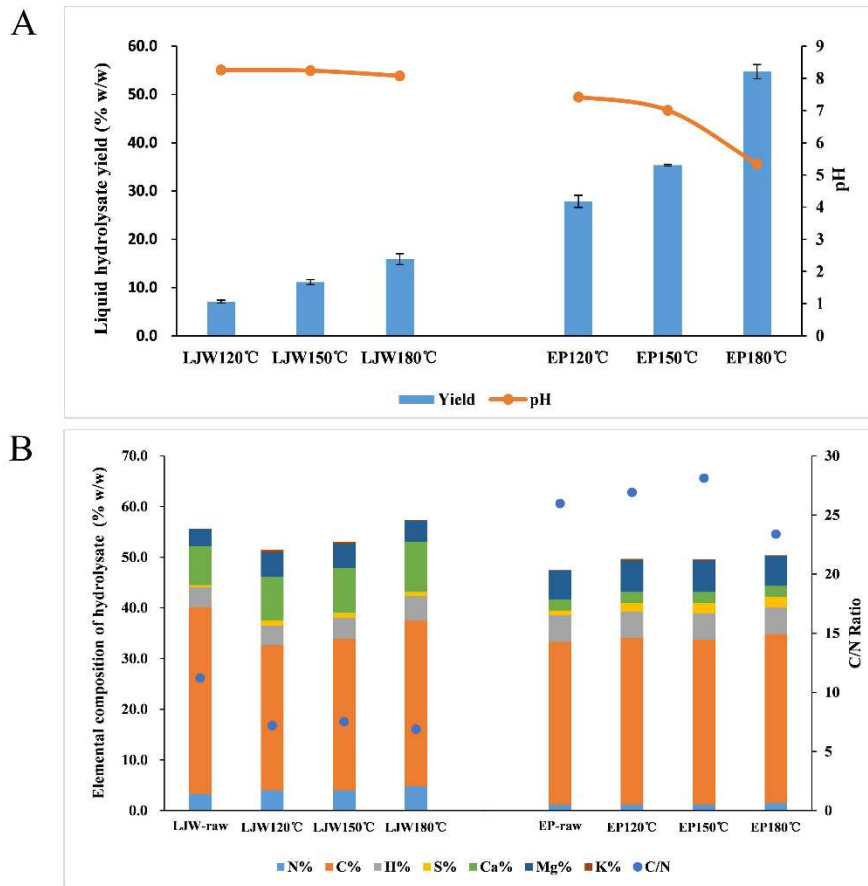
Table 2. Indole-3-acetic acid (IAA) production of strains in different culture media

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Culture medium	Strains	IAA production (ug/mL)
EP hydrolysate	T	2.48±0.21 ^b
	C	3.74±0.23 ^a
	L	0.06±0.01 ^c
LJW hydrolysate	T	1.07±0.42 ^c
	C	0.01±0.01 ^c
	L	1.02±0.36 ^c

514 Different letters in the same column indicate significant difference between different groups (P<0.05).

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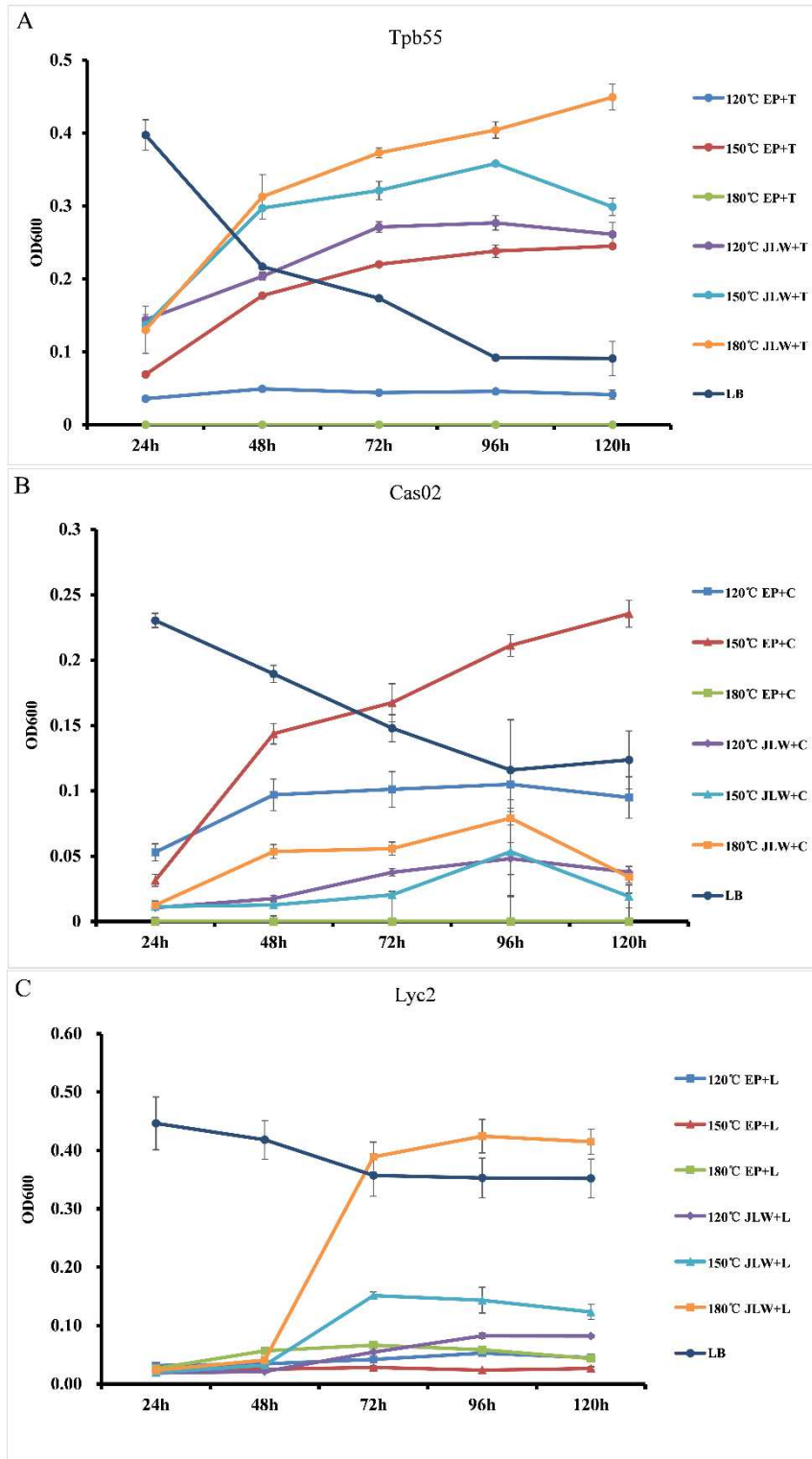


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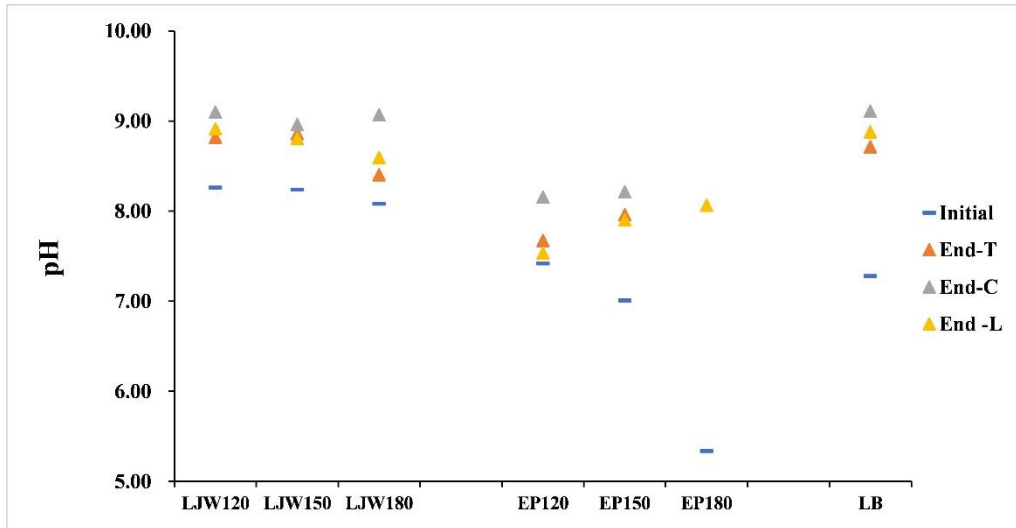
517 Figure 1. Microwave assisted hydrolysis of algae waste biomass. (A) Yields and pH of
 518 hydrolysates from different microwave conditions; (B) Elemental composition and carbo-
 519 nitrogen (C/N) ratio of hydrolysates from different microwave conditions

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 523 Figure 2. Growth of PGPR strains in hydrolysates from different microwave conditions.
 524 (A) *Bacillus subtilis* strain Tpb55; (B) *Bacillus amyloliquefaciens* strain Cas02; (C)
 525 *Burkholderia pyrrocinia* strain Lyc2
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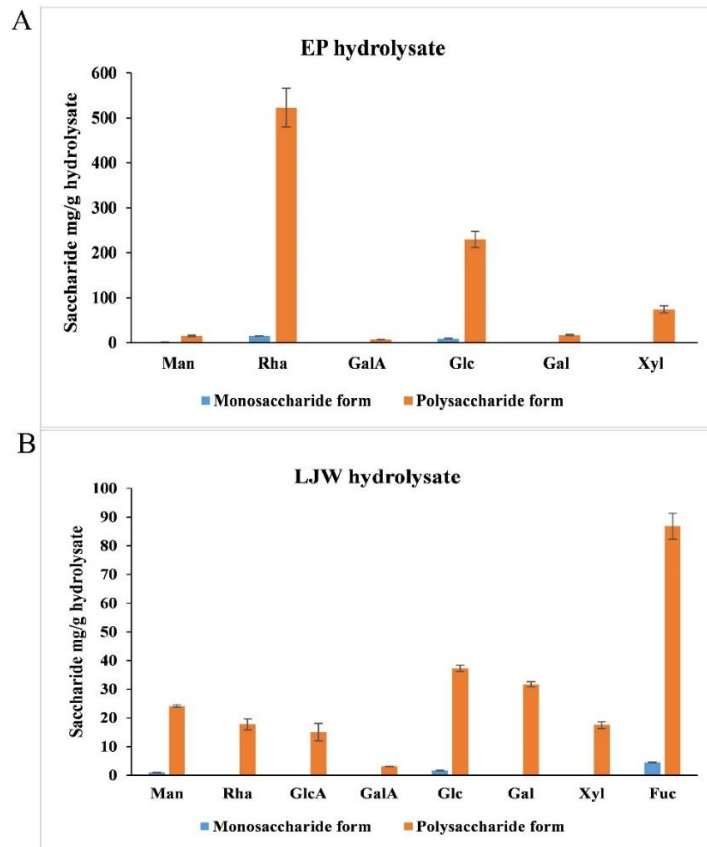
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Figure 3. pH change after 120 h cultivation of strains in different hydrolysates

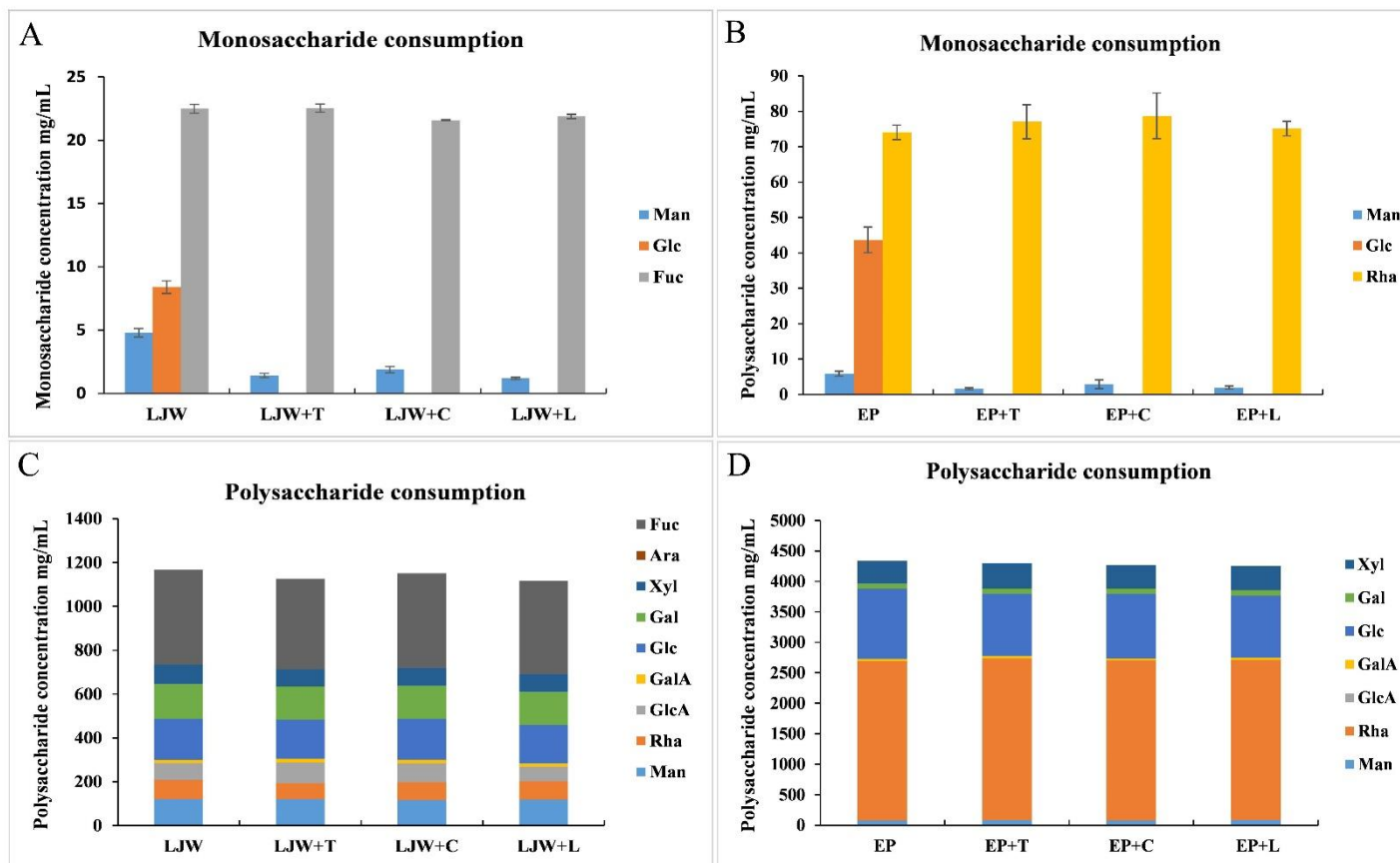


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533 Figure 4. Carbohydrate analysis of (A) EP hydrolysate from microwave assisted hydrolysis

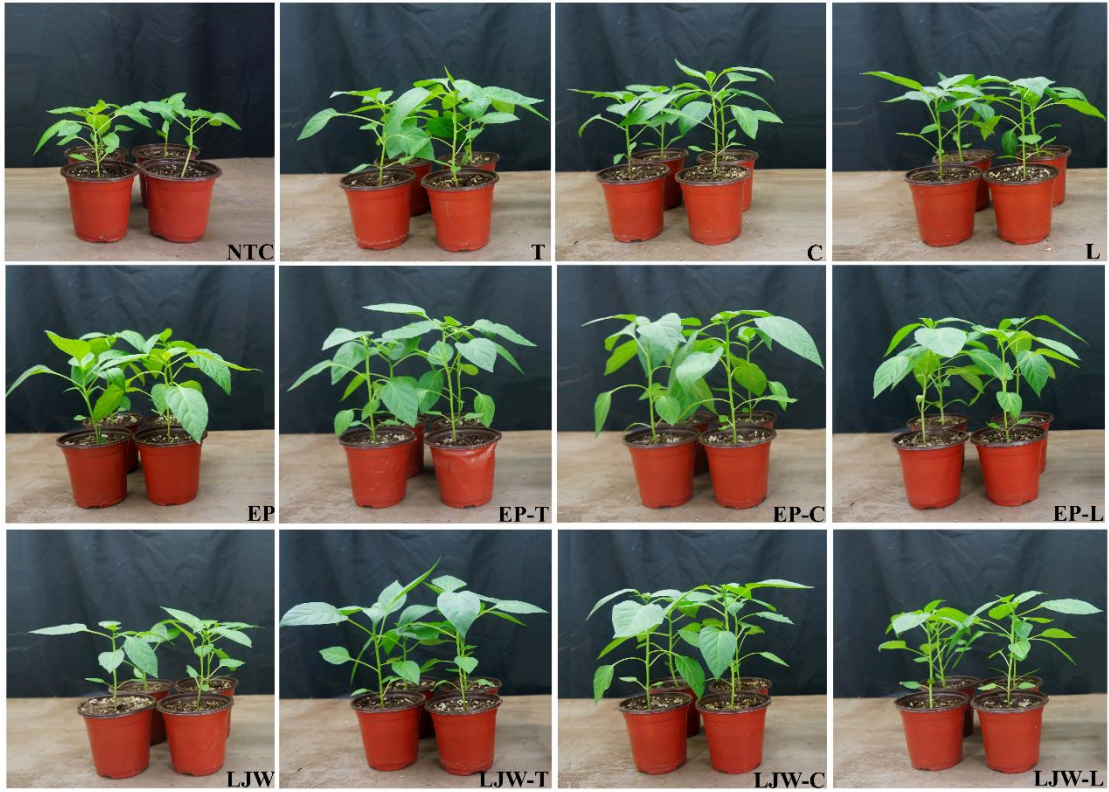
534 at 150 °C; (B) LJW hydrolysate from microwave assisted hydrolysis at 180 °C

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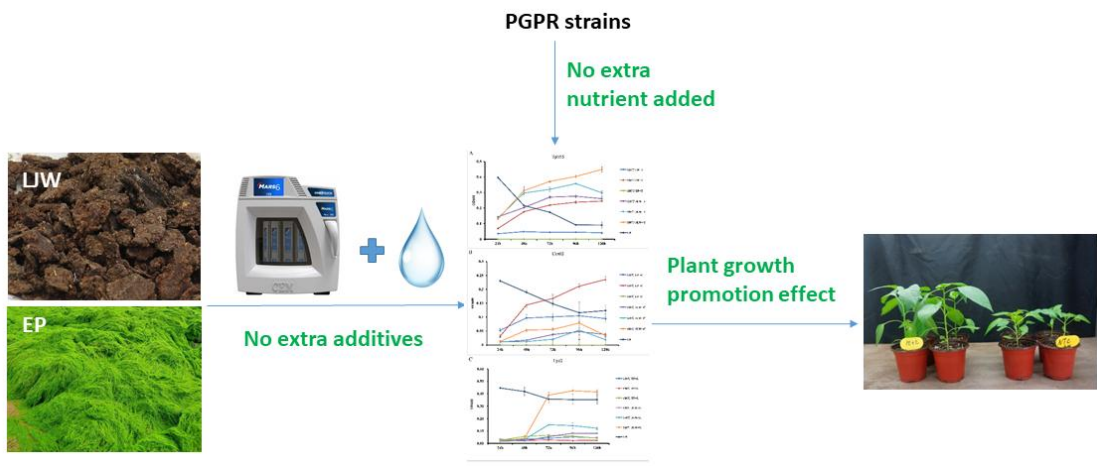
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537 Figure 5. Carbohydrate consumption of PGPR during cultivation in hydrolysates from two algae waste biomass. Monosaccharide consumption of
 538 three strains in (A) LJW hydrolysate from microwave assisted hydrolysis at 180 °C and (B) EP hydrolysate from microwave assisted hydrolysis at
 539 150 °C; Polysaccharide consumption of three strains in (C) LJW hydrolysate from microwave assisted hydrolysis at 180 °C and (B) EP hydrolysate
 540 from microwave assisted hydrolysis at 150 °C



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Figure 6. Effect of different treatments on pepper growth



TableS1. Evaluation of cultivation efficiency of strains under different culture medium

Treatment		Incubate time (96h)						LB
		LJW 120°C	LJW 150°C	LJW 180°C	EP 120°C	EP 150°C	EP 180°C	
Log CFU/ml	Tbp55	7.74	7.71	7.87	7.13	7.40	0	7.27
	Cas02	6.30	6.48	6.51	6.70	6.85	0	6.87
	Lyc2	7.30	7.70	7.88	6.20	6.06	6.22	7.88

Count of viable cells in each treatment at 96h incubation. The strains Tbp55, Cas02 and Lyc2 were incubated in LJW and EP hydrolysate medium, LB was used as control. Pipette each medium separately for gradient dilution, the culture medium with different dilutions were spread on LB solid medium for colony counting. The number of viable cells per ml of culture medium in different treatments is converted into log form for display. Each treatment was repeated 5 times, and the data display in the table is the average of 5 repeats

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: