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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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.

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

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Abstracts

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 *Laminaria japonica* processing waste (LJW) and *Enteromorpha prolifera* (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 (*Bacillus subtilis* strain Tpb55, *Bacillus amyloliquefaciens* strain Cas02, and *Burkholderia pyrrocinia* strain Lyc2) in the two media were investigated. LJW hydrolysate from 180^{o}C and EP hydrolysate from 150^{o}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.

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

Abbreviations

PGPR -- plant growth-promoting rhizobacteria

LJW -- *Laminaria japonica* processing waste

EP -- *Enteromorpha prolifera*

IAA -- indole-3-acetic acid

LB – Luria-Bertani

TFA -- trifluoroacetic acid

NTC – None treatment control

Man – mannose

Rha – rhamnose

GlcA – glucuronic acid

GalA – galacturonic acid

Glc – glucose

Gal – galactose

Xyl – xylose

Fuc – fucose

1. Introduction

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

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

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

2. Materials and reagents

2.1 Materials and reagents

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

2.2 Microwave assisted hydrolysis of algal waste

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

2.3 Microorganisms

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

2.4 PGPR cultivation in the algal waste hydrolysates

The bacterial strains were cultured at 28^{o}C on Luria-Bertani (LB) agar medium. To prepare the seed culture of the strains, single colony of the bacteria was picked up with sterilized bamboo sticks and cultured in LB broth in an orbital shaker at 28 ^{o}C and 175 rpm for 24 h. This pre-culture was adjusted to an optical density OD_{600} of 0.3 with LB medium before inoculation on algal hydrolysates. In shake flasks, 5 g/L algal hydrolysates were inoculated with pre-culture at 0.1% (v/v) of total culture volume. The flasks were sealed with gas-permeable film to avoid evaporation and were incubated at 28 ^{o}C and 175 rpm for 120 h, with readings of OD_{600} performed every 24h. The OD_{600} values of non-inoculated algal hydrolysates were subtracted from the final OD_{600}. The number of viable cells was determined by counting colonies formed on the LB agar plates using the plate dilution method every 24 h. After cultivation, the broth cultures were centrifuged at 7500g for 5min and pass through a sterile microfiltration membrane (0.22-μm pore size) to collect supernatant for further saccharide utilization analysis.

2.5 Seeding growth test

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

via centrifugation at 5000 g for 5 min at room temperature, and pellet was resuspended in cultivation supernatant (for EP hydrolysate- PGPR cultivation mixtures and LJW hydrolysate-PGPR cultivation mixtures) or sterile water (for PGPR suspensions) to a final OD_{600} of 0.3. Algal hydrolysates treatment broths were prepared by dissolving the hydrolysates at 5 g/L without bacterial cultivation.

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

2.7 Analytical methods

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

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

(PerkinElmer, USA).

Indole acetic acid (IAA) production was evaluated as described by (Bric et al., 1991) with slight modifications. Briefly, 100 mL of algal hydrolysates medium (5 g/L) containing 5 mM L-tryptophan was inoculated with 100 µL bacterial suspension (OD=0.3) and incubated at 28 ^{o}C for 96 h in a shaking incubator at 175 rpm. Fully grown cultures were extracted by centrifugation (8000 g; 5 min), and the supernatant (2 mL) was mixed with 4 mL of the Salkowski reagent (1:30:50 ratio of 0.5 M $FeCl_3$/ 98% (w/w) H_2SO_4/ distilled water). The pink color developed was measured at 530 nm. The medium without inoculation of strains was used as the control.

2.8 Statistical analysis

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

3. Results and discussion

3.1 Microwave assisted hydrolysis of algal waste

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

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

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

3.2 Evaluation of algal waste hydrolysates as medium for PGPR cultivation

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

showed different growth patterns. *B. subtilis* strain Tpb55 showed a higher OD value in the LJW hydrolysates than in the EP hydrolysates, and the most suitable culture medium was the LJW hydrolysate from microwave treatment at 180 ^{o}C, with an OD value of 0.45 for120 h cultivation (Figure 2A). While *B. amyloliquefaciens* strain Cas02 preferred EP hydrolysates, especially the one from 150 ^{o}C microwave treatment, showing an OD value of 0.23 for 120 h cultivation (Figure 2B). Similar to strain Tpb55, *B. pyrrocinia* strain Lyc2 had the highest OD value in the LJW hydrolysate from microwave 180 ^{o}C (Figure 2C). Moreover, strains Tpb55 and Cas02 were not able to grow in the slightly acidic EP hydrolysate from 180 ^{o}C, while strain Lyc2 still had a growth curve in that medium, indicating the better acid tolerance of Lyc2. It was also observed that the cell growth of all three strains was achievable in 24 h in LB medium, whereas 24-48 h was required for strains to thrive in the hydrolysates medium. This phenomenon is in consistent with previous studies in which bacterial strain was cultivated in acidic hydrolysates from potato peel waste(Abdelraof et al., 2019). Out of expectation, the maximal OD values of three strains in their favorable hydrolysates medium were all comparable with those in LB medium, which could be considered attractive for future commercialization.

The numbers of viable cells were determined daily in these cultures. The results for 96 h incubation were shown in Table S1, as the highest OD values were obtained in 96 h in most cases for hydrolysates medium. The CFU ml^{-1} values of bacteria were in good agreement with the OD values. For the LJW waste hydrolysates, the CFU ml^{-1} values were similar for strains Tpb55 and Lyc2 (more than 10^{7} CFU mL^{-1}), which were higher than that for strain Cas02 (less than 10^{7} CFU mL^{-1}). For the EP hydrolysates, the CFU ml^{-1} values for the three strains were in the order of Tpb55>Cas02>Lyc2. Additionally, a pH increase to slightly basic value was observed in all cases (Figure 3), indicating that bacteria metabolized proteins and amino acids, releasing NH_4^+ into the medium(Abeln et al., 2019).

The results demonstrate that microwave processing without any catalytic addition can

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

3.3 Carbohydrate utilization during cultivation

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

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

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

3.4 Effect of hydrolysates-PGPR cultivation mixture on pepper growth

To investigate whether the hydrolysates-PGPR cultivation mixture can be jointly applied for promoting plant growth, 12 treatments were conducted on pepper seedlings. Agricultural parameters including stem height, leaf number, maximum leaf length, maximum leaf width, and dry weight of biomass were measured (Table 1). The images of plants after treatment are shown in Figure 6. As can be seen, application of three strains alone all showed promotions on pepper growth, but generally without significant difference compared with the H_2O control, except for the leaf number of Tpb55, and biomass dry weight of Tpb55 and Lyc2 ($P<0.05$). When applied hydrolysates-PGPR cultivation mixture, the EP hydrolysate-PGPR cultivation mixtures performed much better than the LJW hydrolysate-PGPR cultivation mixture, probably because of the higher polysaccharide content which could promote the growth of the pepper seedlings as well(El Modafar et al., 2012). Although the LJW hydrolysate-PGPR cultivation mixtures exhibited a positive effect on pepper growth compared with the H_2O control, there was no significant difference compared with the PGPR only treatments. However, peppers treated by the EP hydrolysate-PGPR cultivation mixtures not only had significantly better growth than H_2O control in terms of all parameters measured ($P<0.05$), but also showed an increased growth trend compared with PGPR only treatments. Recent study demonstrated that plant polysaccharides (e.g. pectin, xylan, etc.) can serve as a carbon source used to produce the extracellular matrix and can act as an environmental cue that triggers biofilm formation (Beauregard et al., 2013), thereby, stimulating the colonization of PGPR, which is one of key factors determining the performance of the

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

In particular, the EP hydrolysate-Cas02 cultivation mixture showed the best plant promoting effect among all treatments, with stem height, leaf number, maximum leaf length, maximum leaf width, and dry weight being increased by 136%, 131%, 131%, 128%, and 219%, respectively, compared with the H_2O control. Nevertheless, the promotion effect of EP hydrolysate-Cas02 treatment was significantly higher ($P<0.05$) than that of Cas02 alone, EP hydrolysate alone, or H_2O control.

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

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

4. Conclusion

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

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgement

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

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Tables and Figures

Table 1. Growth indices of pepper with different treatment

Treatment	Stem height /mm	Leaf number	Maximum leaf length/mm	Maximum leaf width/mm	Dry weight /mg
H_2O 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}

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

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]

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

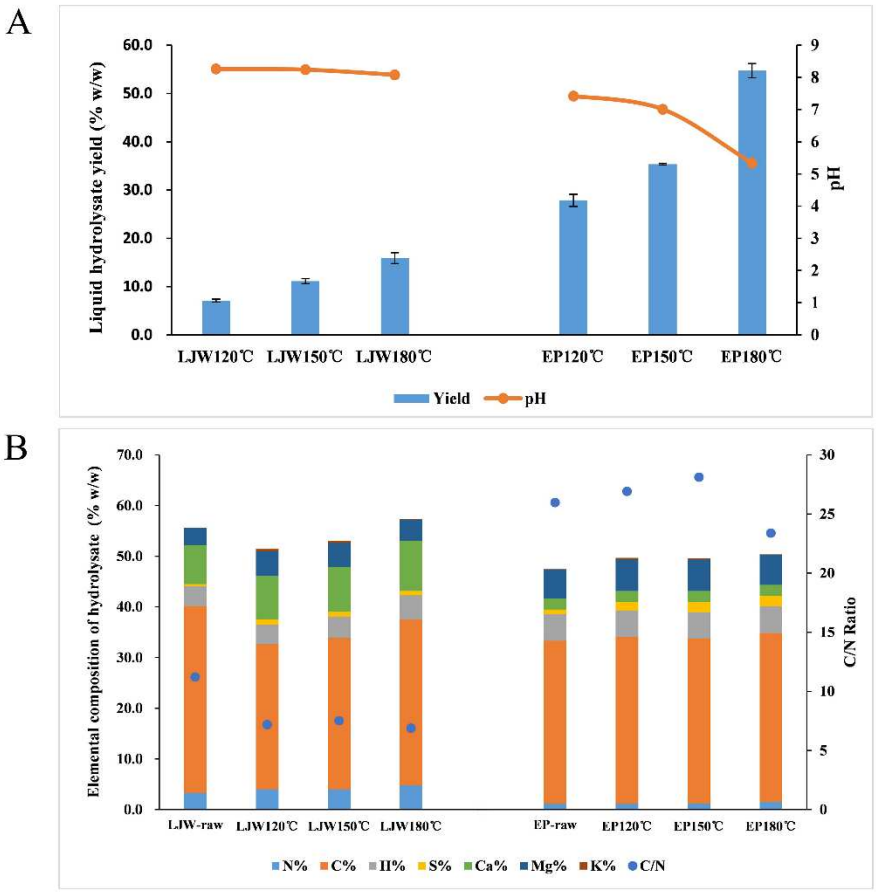

Figure 1. Microwave assisted hydrolysis of algae waste biomass. (A) Yields and pH of hydrolysates from different microwave conditions; (B) Elemental composition and carbo-nitrogen (C/N) ratio of hydrolysates from different microwave conditions

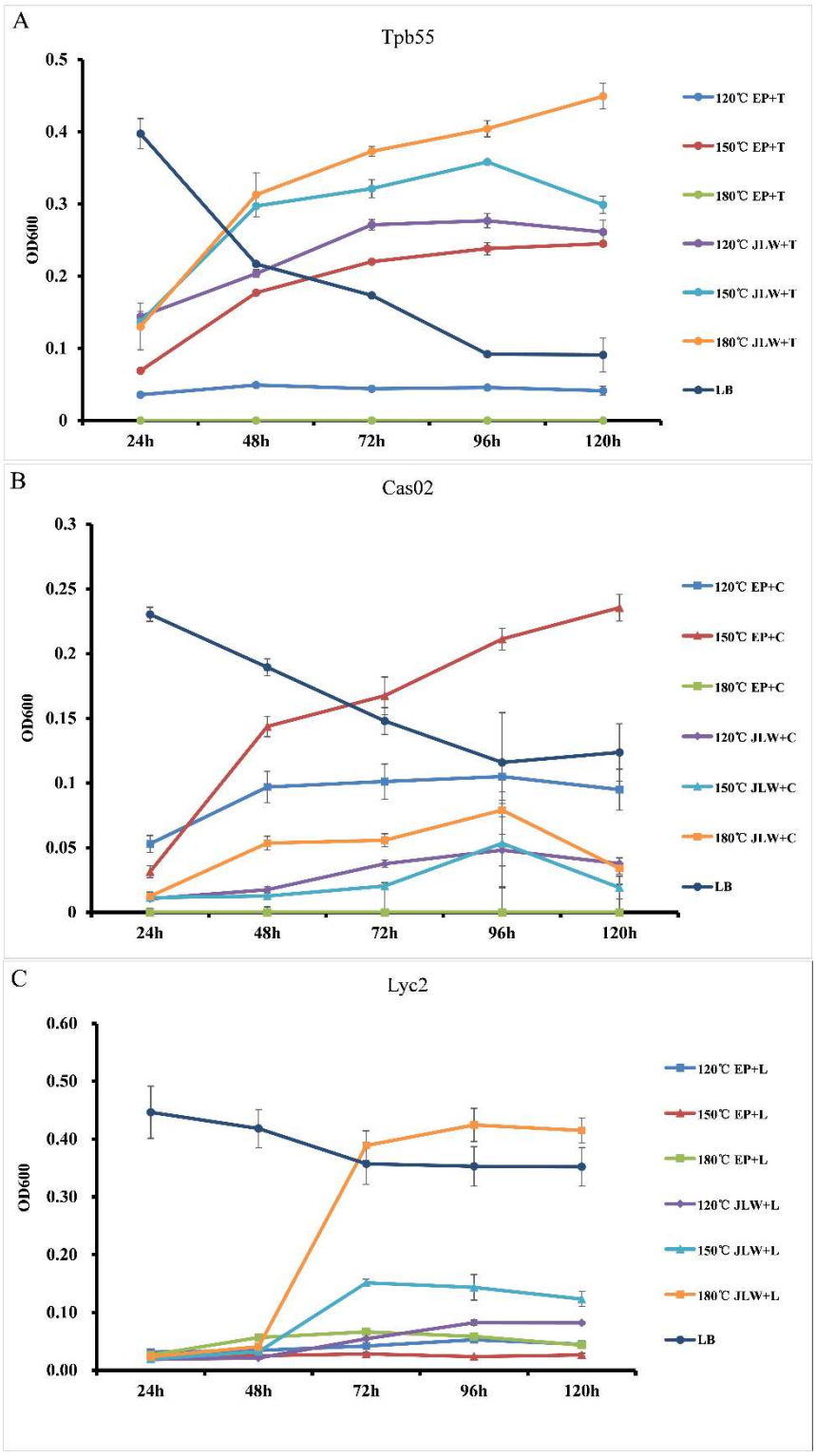

Figure 2. Growth of PGPR strains in hydrolysates from different microwave conditions. (A) *Bacillus subtilis* strain Tpb55; (B) *Bacillus amyloliquefaciens* strain Cas02; (C) *Burkholderia pyrrocinia* strain Lyc2

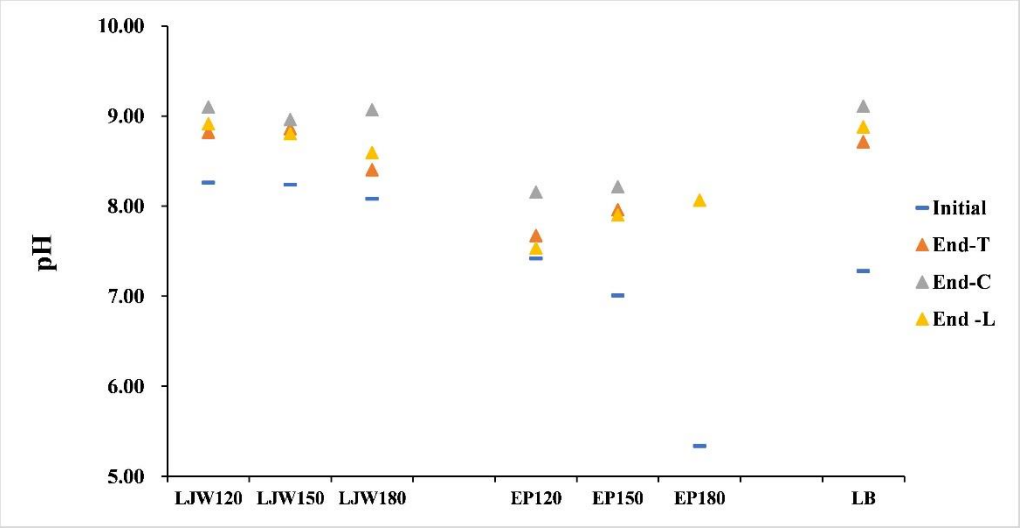

Figure 3. pH change after 120 h cultivation of strains in different hydrolysates

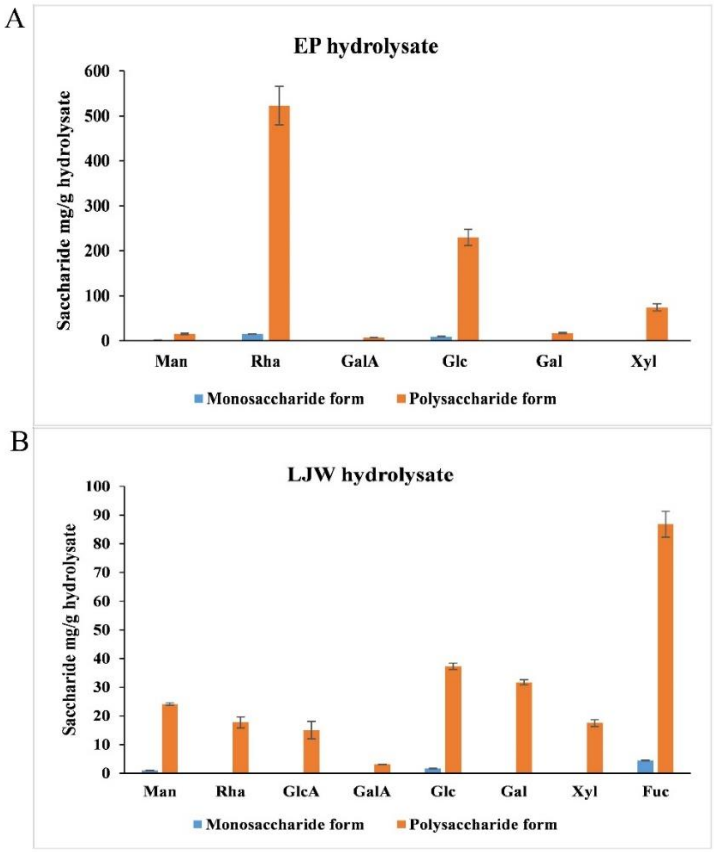

Figure 4. Carbohydrate analysis of (A) EP hydrolysate from microwave assisted hydrolysis at 150 ^{o}C; (B) LJW hydrolysate from microwave assisted hydrolysis at 180 ^{o}C

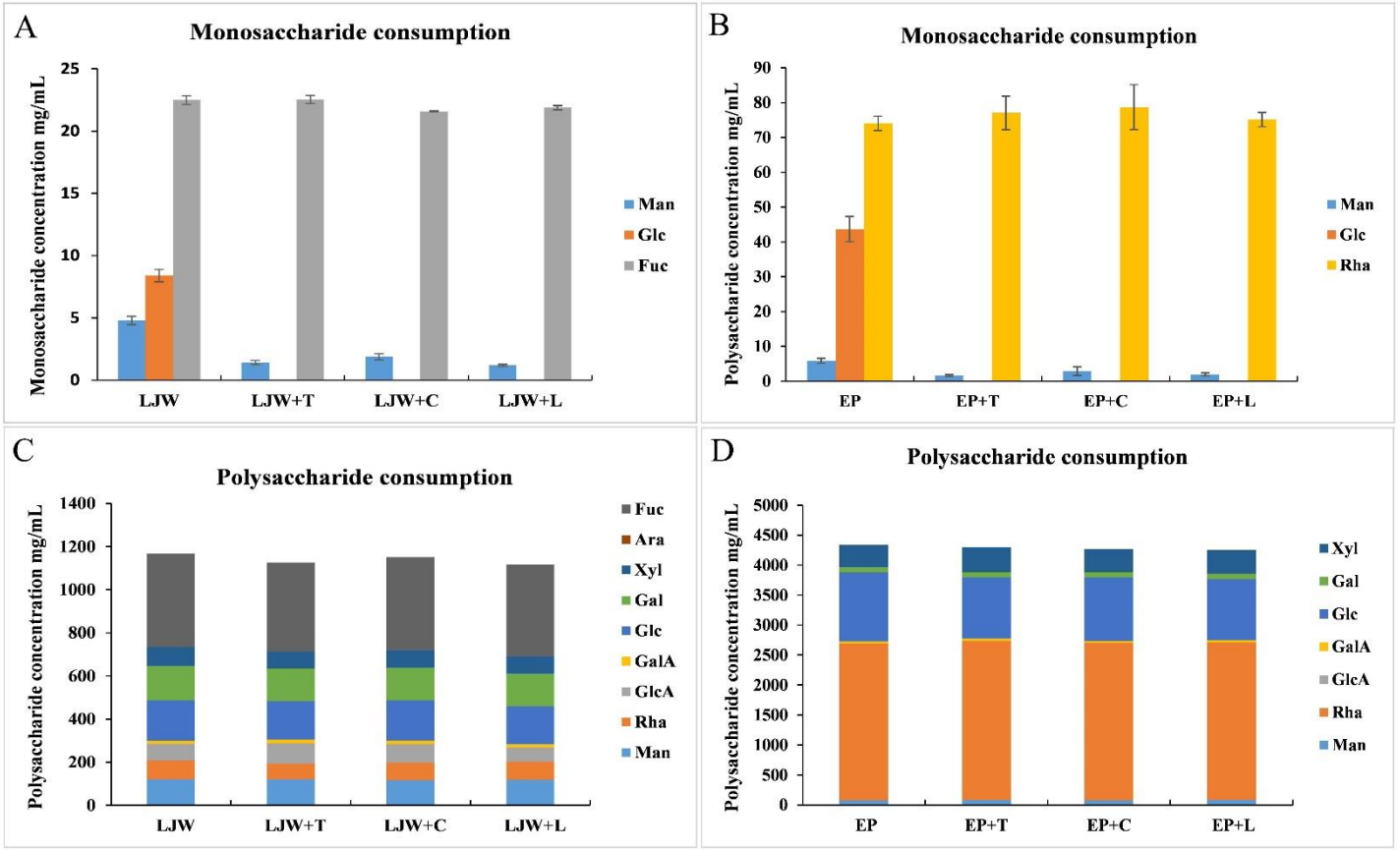

Figure 5. Carbohydrate consumption of PGPR during cultivation in hydrolysates from two algae waste biomass. Monosaccharide consumption of three strains in (A) LJW hydrolysate from microwave assisted hydrolysis at 180 °C and (B) EP hydrolysate from microwave assisted hydrolysis at 150 °C; Polysaccharide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

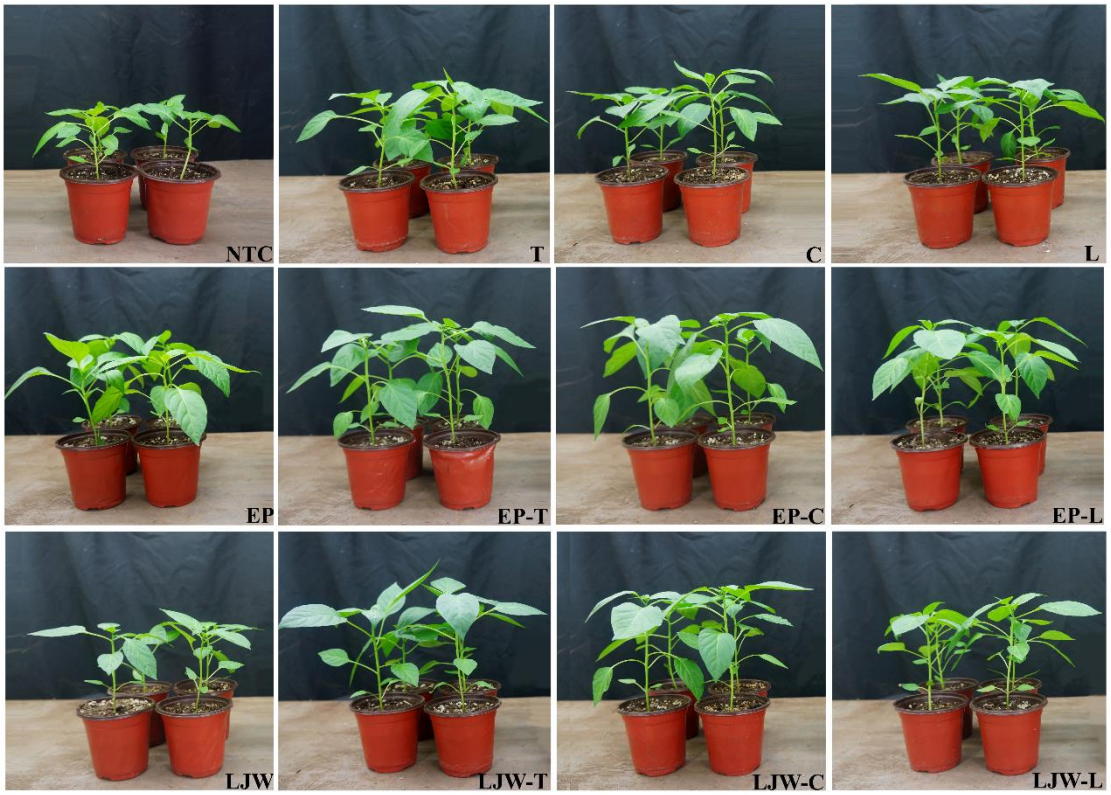

Figure 6. Effect of different treatments on pepper growth

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

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Abstracts

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 *Laminaria japonica* processing waste (LJW) and *Enteromorpha prolifera* (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 (*Bacillus subtilis* strain Tpb55, *Bacillus amyloliquefaciens* strain Cas02, and *Burkholderia pyrrocinia* 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 ~~on~~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 ~~opens a new avenue of~~ 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.

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

Abbreviations

PGPR -- plant growth-promoting rhizobacteria

LJW -- *Laminaria japonica* processing waste

EP -- *Enteromorpha prolifera*

IAA -- indole-3-acetic acid

LB – Luria-Bertani

TFA -- trifluoroacetic acid

NTC – None treatment control

Man – mannose

Rha – rhamnose

GlcA – glucuronic acid

GalA – galacturonic acid

Glc – glucose

Gal – galactose

Xyl – xylose

Fuc – fucose

1. Introduction

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

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

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

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

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

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

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

2. Materials and reagents

2.1 Materials and reagents

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

2.2 Microwave assisted hydrolysis of algal waste

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

2.3 Microorganisms

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

2.4 PGPR cultivation in the algal waste hydrolysates

The bacterial strains were cultured at 28°C on Luria-Bertani (LB) agar medium. To prepare the seed culture of the strains, single colony of the bacteria was picked up with sterilized bamboo sticks and cultured in LB broth in an orbital shaker at 28 °C and 175 rpm for 24 h. This pre-culture was adjusted to an optical density OD_{600} of 0.3 with LB medium before inoculation on algal hydrolysates. In shake flasks, 5 g/L algal hydrolysates were inoculated with pre-culture at 0.1% (v/v) of total culture volume. The flasks were sealed with gas-permeable film to avoid evaporation and were incubated at 28 °C and 175 rpm for 120 h, with readings of OD_{600} performed every 24h. The OD_{600} values of non-inoculated algal hydrolysates were subtracted from the final OD_{600}. The number of viable cells was determined by counting colonies formed on the LB agar plates using the plate dilution method every 24 h. After cultivation, the broth cultures were centrifuged at 7500g for 5min and pass through a sterile microfiltration membrane (0.22-μm pore size) to collect supernatant for further saccharide utilization analysis.

2.5 Seeding growth test

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

for 96 h. Bacterial cells were collected via ~~certification~~ centrifugation at 5000 g for 5 min at room temperature, and pellet was resuspended in cultivation supernatant (for EP hydrolysate- PGPR cultivation mixtures and LJW hydrolysate-PGPR cultivation mixtures) or sterile water (for PGPR suspensions) to a final OD_{600} of 0.3. Algal hydrolysates treatment broths were prepared by dissolving the hydrolysates at 5 g/L without bacterial cultivation.

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

2.7 Analytical methods

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

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

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

Indole acetic acid (IAA) production was evaluated as described by (Bric et al., 1991) with slight modifications. Briefly, 100 mL of algal hydrolysates medium (5 g/L) containing 5 mM L-tryptophan was inoculated with 100 µL bacterial suspension (OD=0.3) and incubated at 28 ^{o}C for 96 h in a shaking incubator at 175 rpm. Fully grown cultures were extracted by centrifugation (8000 g; 5 min), and the supernatant (2 mL) was mixed with 4 mL of the Salkowski reagent (1:30:50 ratio of 0.5 M $FeCl_3$/ 98% (w/w) H_2SO_4/ distilled water). The pink color developed was measured at 530 nm. The medium without inoculation of strains was used as the control.

2.8 Statistical analysis

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

3. Results and discussion

3.1 Microwave assisted hydrolysis of algal waste

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

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

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

3.2 Evaluation of algal waste hydrolysates as medium for PGPR cultivation

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

sources for cell cultivation. The growth of bacteria~~l~~ in hydrolysates was assessed by reading OD values and counting viable cells. The results showed that all three strains could grow in the hydrolysates of both algal waste without any extra nutrient added, but showed different growth patterns. *B. subtilis* strain Tpb55 showed a higher OD value in the LJW hydrolysates than in the EP hydrolysates, and the most suitable culture medium was the LJW hydrolysate from microwave treatment at 180 ^{o}C, with ~~the~~ an OD value of 0.45 for120 h cultivation (Figure 2A). While *B. amyloliquefaciens* strain Cas02 preferred EP hydrolysates, especially the one from 150 ^{o}C microwave treatment, showing ~~the~~ an OD value of 0.23 for 120 h cultivation (Figure 2B). Similar ~~with~~ to strain Tpb55, *B. pyrrocinia* strain Lyc2 had the highest OD value in the LJW hydrolysate from microwave 180 ^{o}C (Figure 2C). Moreover, strains Tpb55 and Cas02 were not ~~be~~ able to grow in the slightly acidic EP hydrolysate from 180 ^{o}C, while strain Lyc2 still had a growth curve in that medium, indicating the better acid tolerance of Lyc2. It ~~is~~ was also observed that the cell growth of all three strains was achievable in 24 h in LB medium, whereas 24-48 h was ~~needed~~ required for strains to ~~get used to~~thrive in the hydrolysates medium. This phenomenon is in ~~accordance~~ consistent with previous studies in which bacterial strain was cultivated in acidic hydrolysates from potato peel waste(Abdelraof et al., 2019). Out of expectation, the maximal OD values of three strains in their favorable hydrolysates medium were all comparable with those in LB medium, which could be considered attractive for future commercialization.

The numbers of viable cells were determined daily in these cultures. The results for 96 h incubation were shown in Table S1, as the highest OD values were obtained in 96 h in most cases for hydrolysates medium. ~~As can be seen, the~~ The CFU ml^{-1} values of bacteria~~l~~ were in good agreement with the OD values. For the LJW waste hydrolysates, the CFU ml^{-1} values were similar for strains Tpb55 and Lyc2 (more than 10^{7} CFU mL^{-1}), which were higher than that for strain Cas02 (less than 10^{7} CFU mL^{-1}). For the EP hydrolysates, the CFU ml^{-1} values for the three strains were in the order of Tpb55>Cas02>Lyc2. Additionally, a pH increase to slightly basic value was observed in all cases (Figure 3), indicating that

bacteria~~l~~ metabolized proteins and amino acids, releasing NH_4^+ into the medium(Abeln et al., 2019).

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

3.3 Carbohydrate utilization during cultivation

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

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

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

3.4 Effect of hydrolysates-PGPR cultivation mixture on pepper growth

To investigate whether the hydrolysates-PGPR cultivation mixture can be jointly applied for promoting plant growth, 12 treatments were conducted on pepper seedlings. Agricultural parameters including stem height, leaf number, ~~maximal~~ maximum leaf length, maximum~~al~~ leaf width, and dry weight of biomass were measured (Table 1). The images of plants after treatment ~~were~~ are shown in Figure 6. As can be seen, application of three strains alone all showed promotions on pepper growth, but generally without significant difference compared with the H_2O control, except for the leaf number of Tpb55, and biomass dry weight of Tpb55 and Lyc2 ($P<0.05$). When applied hydrolysates-PGPR cultivation mixture, the EP hydrolysate-PGPR cultivation mixtures performed much better than the LJW hydrolysate-PGPR cultivation mixture, probably because of the higher polysaccharide content which could promote the growth of the pepper seedlings as well(El Modafar et al., 2012). Although the LJW hydrolysate-PGPR cultivation mixtures exhibited a positive effect on pepper growth compared with the H_2O control, there ~~is~~ was no significant difference compared with the PGPR only treatments. However, peppers treated by the EP hydrolysate-PGPR cultivation mixtures not only had

significantly better growth than H_2O control in terms of all parameters measured ($P<0.05$), but also showed an ~~obviously~~ increased growth trend compared with PGPR only treatments. Recent~~ly~~ study demonstrated that plant polysaccharides (e.g. pectin, xylan, etc.) can serve as a carbon source used to produce the extracellular matrix and can act as an environmental cue that triggers biofilm formation (Beauregard et al., 2013), ~~therefore~~thereby, stimulating the colonization of PGPR, which is one of key factors determining the performance of the bacterium (Xu et al., 2017). This is probably the reason that the ~~why~~ EP hydrolysate-PGPR cultivation mixtures that contained a high content of polysaccharide showed better plant growth-~~-~~promoting~~on~~ effects.

In particular, the EP hydrolysate-Cas02 cultivation mixture showed the best plant promoting effect among all treatments, ~~of which the~~with stem height, leaf number, ~~maximal~~ maximum leaf length, ~~maximal~~ maximum leaf width, and dry weight being increased by 136%, 131%, 131%, 128%, and 219%, respectively, compared with the H_2O control. Nevertheless, the promotion effect of EP hydrolysate-Cas02 treatment was significantly higher ($P<0.05$) than that of Cas02 alone, EP hydrolysate alone, ~~and~~ or H_2O control.

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

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

4. Conclusion

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

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgement

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

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Tables and Figures

Table 1. Growth indices of pepper with different treatment

Treatment	Stem height /mm	Leaf number	Maximumal leaf length/mm	Maximumal leaf width/mm	Dry weight /mg
H_2O 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}

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

509
510
511
512 **Table 2. Indole-3-acetic acid (IAA) production of strains in different culture media**
513

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).

515

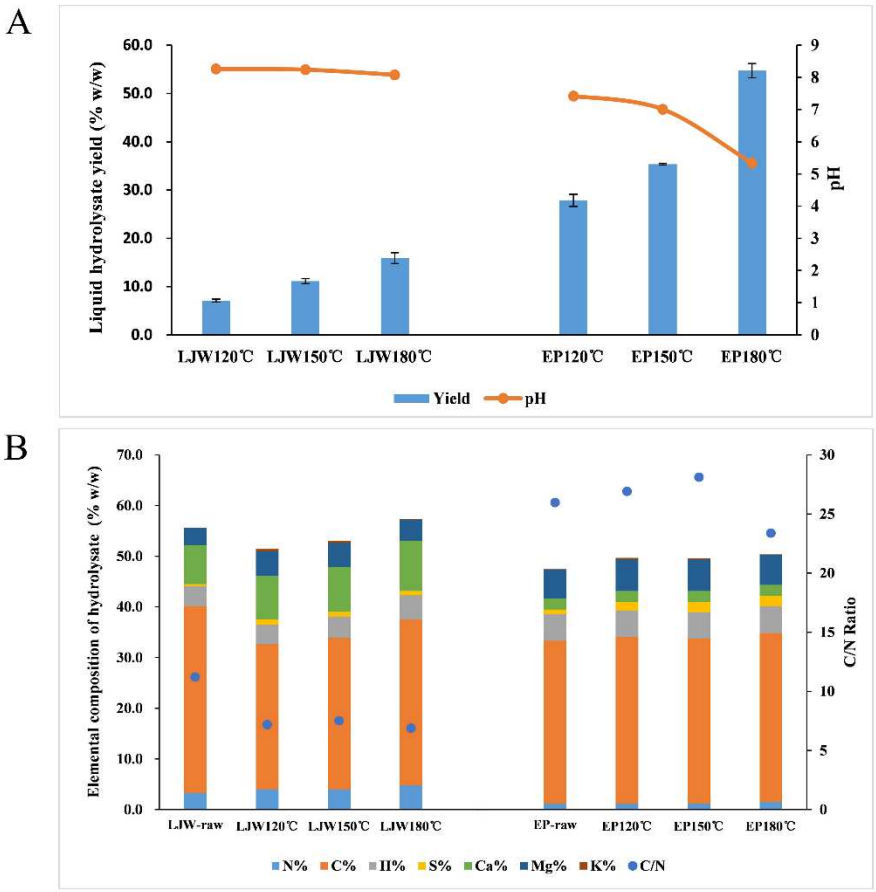


Figure 1. Microwave assisted hydrolysis of algae waste biomass. (A) Yields and pH of hydrolysates from different microwave conditions; (B) Elemental composition and carbo-nitrogen (C/N) ratio of hydrolysates from different microwave conditions

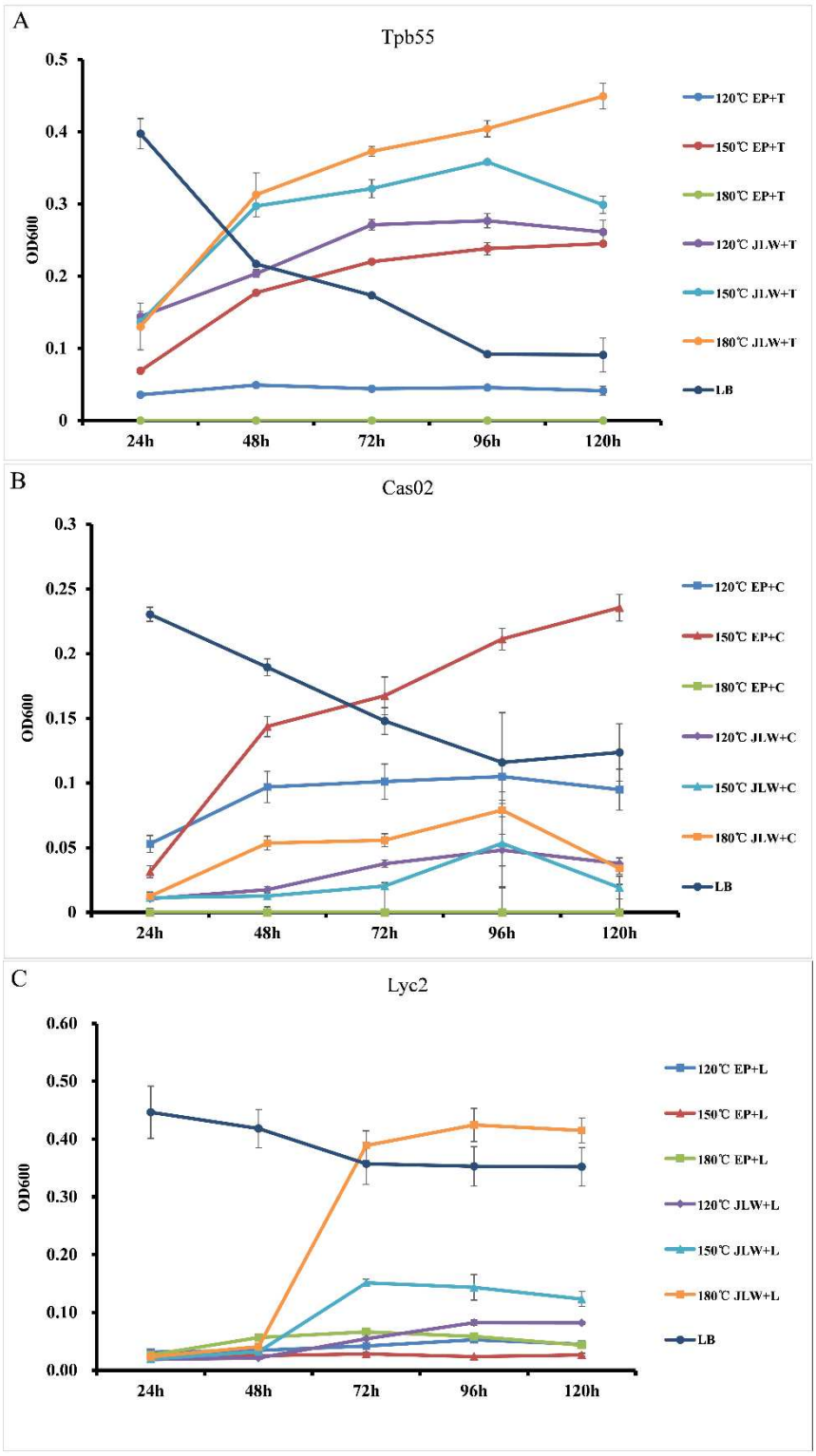
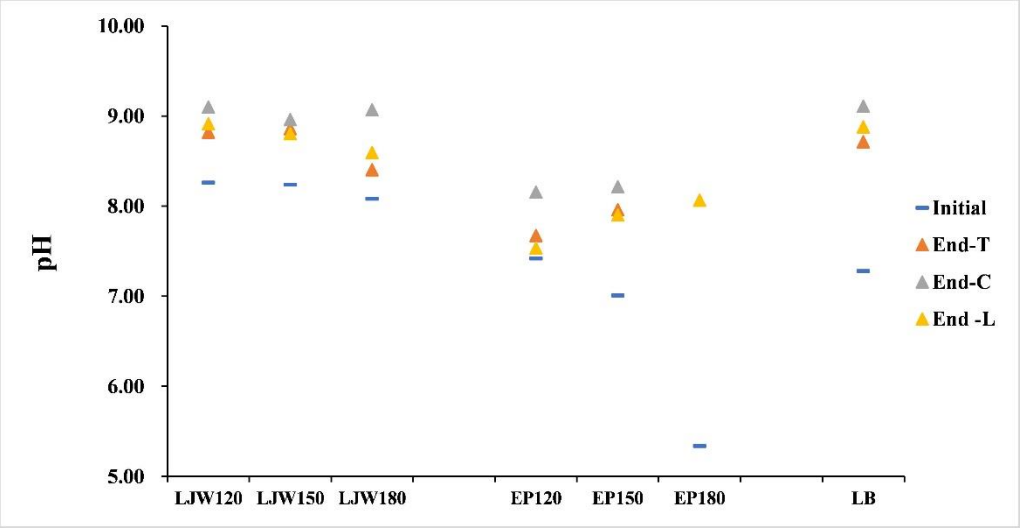


Figure 2. Growth of PGPR strains in hydrolysates from different microwave conditions. (A) *Bacillus subtilis* strain Tpb55; (B) *Bacillus amyloliquefaciens* strain Cas02; (C) *Burkholderia pyrrocinia* strain Lyc2



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

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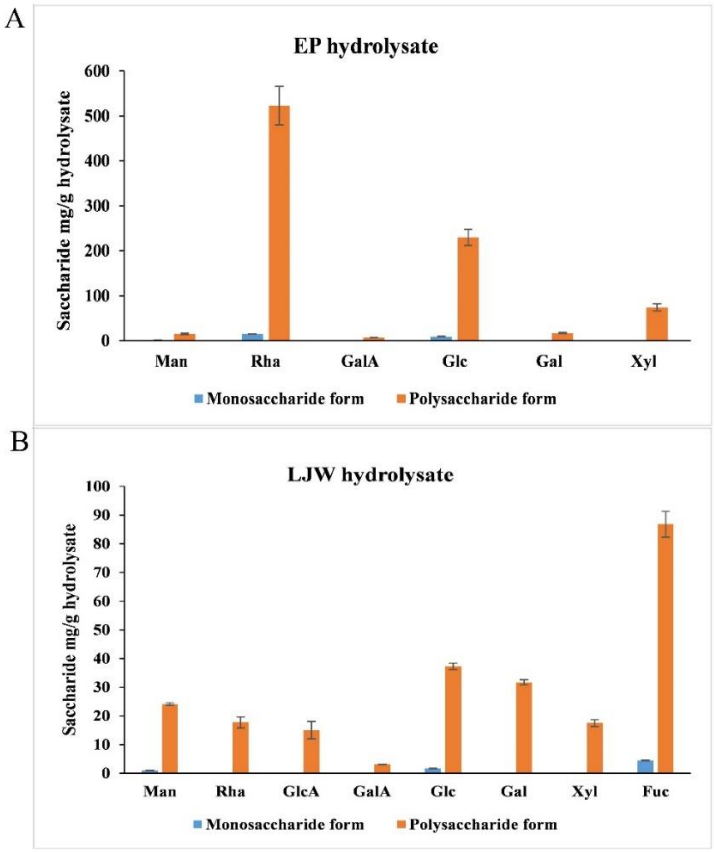

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533 Figure 4. Carbohydrate analysis of (A) EP hydrolysate from microwave assisted hydrolysis
534 at 150 ^{o}C; (B) LJW hydrolysate from microwave assisted hydrolysis at 180 ^{o}C

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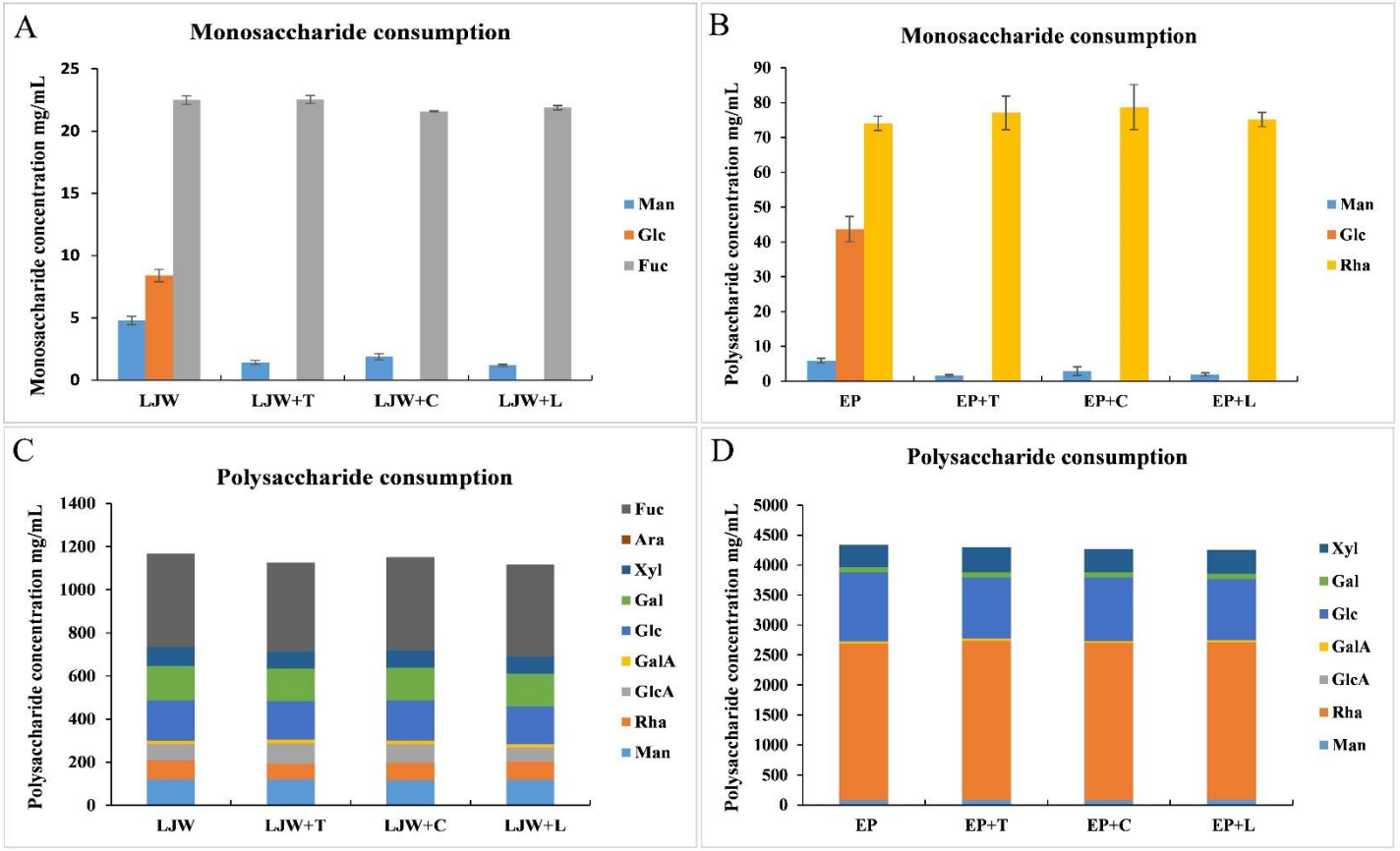


Figure 5. Carbohydrate consumption of PGPR during cultivation in hydrolysates from two algae waste biomass. Monosaccharide consumption of three strains in (A) LJW hydrolysate from microwave assisted hydrolysis at 180 °C and (B) EP hydrolysate from microwave assisted hydrolysis at 150 °C; Polysaccharide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

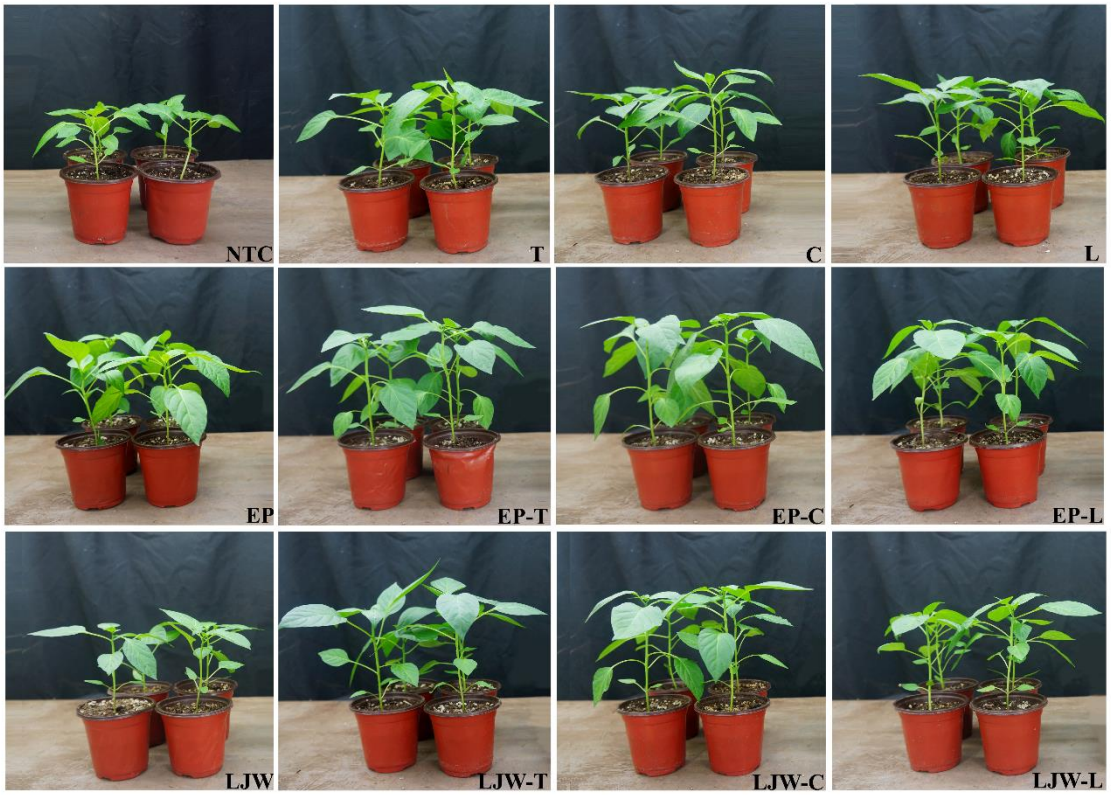
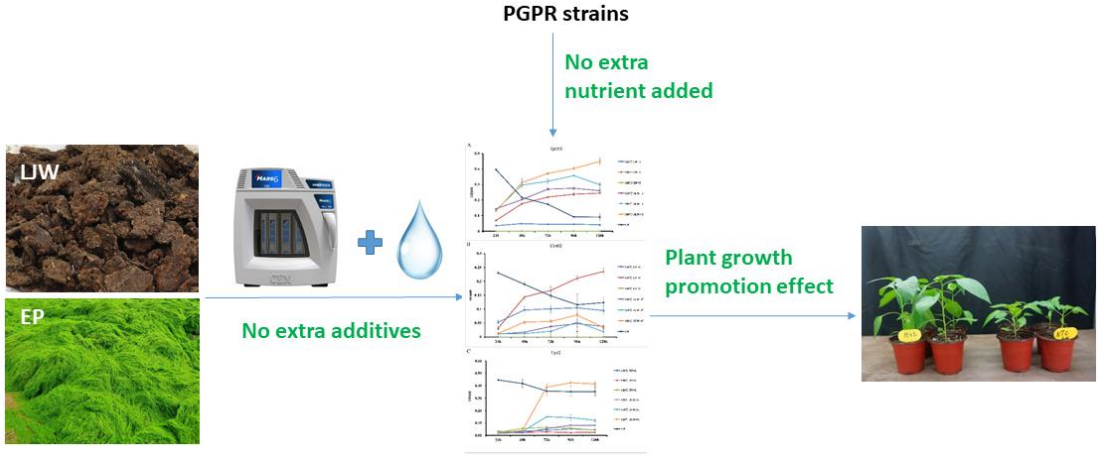


Figure 6. Effect of different treatments on pepper growth

Graphic abstract



Supplementary Interactive Plot Data (CSV)

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

Treatment		Incubate time (96h)						
		LJW 120°C	LJW 150°C	LJW 180°C	EP 120°C	EP 150°C	EP 180°C	LB
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 Interest Statement

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: