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Utilization of Seaweed (*Gracilaria* sp.) Liquid as Cost-Effective Macronutrients and Micronutrients for Bioethanol Production

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Seaweed liquid waste from filter-squeezed drying process of *Gracilaria* sp. causes an unpleasant odour to the environment. Although this waste can partially be used as biofertilizer and heavy metals adsorbent, a bigger portion of the waste remains unutilized thus causing a problem to the environment. The present study aimed to utilize seaweed liquid waste as a supplement in fermentation media. To achieve this aim, *Saccharomyces cerevisiae* as a famous bioethanol producer was used to examine the ability of seaweed liquid to supply macronutrients and micronutrients in the media to produce ethanol. Interestingly, when used as a supplement in the fermentation media to produce ethanol, the liquid boosted ethanol production three folds from 200 mg/L of 2 % glucose alone to 600 mg/L of 2 % glucose in liquid. Additionally, the liquid was able to substitute yeast extract and peptone within YPD media to produce 647.48 mg/L ethanol as compared to only 542.39 mg/L with normal YPD which contains yeast extract and peptone. For that reason, the utilization of seaweed liquid as the supplement in the medium should be considered as an alternative cost-effective media for enhancing ethanol production without the addition of other nutrients.

1. Introduction

Red seaweed, *Gracilaria* sp. is commonly consumed for agar production which contributes to more than 50% of agar production in the world (Baweja et al., 2016). This agar has been commercialized in various industries such as cosmetics, pharmaceuticals, nutraceuticals and food (Jiang et al., 2022). These explorations of red seaweed usage in many fields have increased the demand for red seaweed in the industry sector (Thau et al., 2011). Usually, this seaweed is dried first before they are transported to the relevant industry unless the industry requested fresh seaweed (Francavilla et al., 2013). The conventional methods that are typically used to dry the seaweed are hanging and platform methods (Sade and Ali, 2006). These methods normally require five days or a week during the rainy season to dry the seaweed. A new technique for drying the seaweed has been implemented to meet the demand for dry seaweed.

The new and simple method to dry the seaweed is by rupturing the seaweed and filter-pressed them to separate the liquid from the solid part. This new process helps the farmer to reduce the seaweed drying time and utilize the liquid filtrate for other useful ways such as fertilizer (Eswaran et al., 2005). However, extensive and long-term consumption of this fertilizer could cause adverse effects due to the accumulation of nutrients, salts and heavy metals toward plant growth and human health and indirectly has decreased the interest of farmers in this fertilizer (Carvajal-Muñoz and Carmona-Garcia, 2012). Thus, it is necessary to find an alternative utilization of

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the liquid waste. According to Yun et al. (2022), the seaweed waste is expected to exceed 50 % of raw seaweed biomass.

In general, the red seaweed consists of polysaccharides, proteins, lipids and a low concentration of lignin (Karunakaran and Gurusamy, 2011). In fact, *Gracilaria* sp. is a type of seaweed that contains monosaccharides such as 2-O-methyl-3,6-anhydro-α-L-galactose, 6-O-methyl-β-D-galactose, galactose and glucose together with amino acid (Kazir et al., 2019). Hypothetically, the liquid (filtrate) may contain the similar macronutrient (carbon, nitrogen, phosphorus and sulfur) and micronutrients (other minerals and vitamins) which are interesting to explore as a new medium for bioethanol production.

Recently, Malaysia has upgraded its target of renewable energy in the country's energy mix from 20 % to 31 % by 2025 (Energy, 2021). Hence, the production of bioethanol from indigenous resources could help Malaysia to achieve its target. To the best of our knowledge, the information on the potentiality of seaweed liquid from drying process as supplement in fermentation in producing bioethanol is limited. Therefore, aim of our study is to utilize the liquid byproduct from *Gracilaria* sp. as a new promising nutrient in fermentation medium with existence of either galactose or glucose for bioethanol production via *Saccharomyces cerevisiae*.

2. Materials and methods

Fresh seaweed (*Gracilaria* sp.) was collected from local seaweed farmer in Northern part of Malaysia, and ethanol (99.8%, Fisher Scientific) was supplied locally.

2.1 Inoculum

S. cerevisiae was cultured on potato dextrose agar (PDA) and incubated at 30 °C for 48 h. A single colony of *S. cerevisiae* was transferred into 250 mL of YPD broth (10.0 g/L yeast extract, 20.0 g/L peptone and 20.0 g/L dextrose) and incubated at 30 °C, 150 rpm for 24 h. The cell concentration in the inoculum was adjusted to 5×10^6 cells per mL.

2.2 Seaweed liquid and fermentation media

Seaweed liquid was prepared by referring to Eswaran et al. (2005) with some modifications. The fresh seaweed (*Gracilaria sp.*) was rinsed with tap water to remove impurities and dried at room temperature. Next, 368 g of the seaweed was blended in 1,000 mL distilled water and then filtered through 0.45 µm filter paper. The accumulated filtrates were later considered and mentioned as seaweed liquid. Next, 2 %, 4 % and 6 % (w/v) galactose and glucose media with combination of seaweed liquid were prepared by autoclaving specific volume of seaweed liquid in 250 mL Erlenmeyer flasks and 40 % (w/v) galactose or 40 % (w/v) glucose stock.

2.3 Fermentation

About 7.5 mL of inoculums were added aseptically into 75 mL of fermentation media in 250 mL Erlenmeyer flasks and incubated at 30 °C and 150 rpm for 18 h. The samplings were done after 6 h incubation and continued to the next of every 3 h until 18 h. Three mL of samples were taken for each sampling time and centrifuged at 7,500 rpm for 15 min and filtered via 0.2 µm syringe filter into Gas Chromatography vials. Then, ethanol content in these samples was analyzed by Gas Chromatography.

2.4 Optimum concentration of seaweed liquid as supplement nutrients for ethanol production

In determining the optimum seaweed liquid concentration as the supplement, the concentration of reducing sugar in fermentation media was standardized to 2 % (w/v) glucose by diluting the 40 % (w/v) glucose stock with seaweed liquid as mentioned in Table 1. Prior to the dilution, the amount of reducing sugar in seaweed liquid was quantified by dinitrosalicylic acid (DNS) method. The seaweed liquid and 40 % (w/v) glucose stock were autoclaved at 121 °C for 20 min and filled in the 250 mL Erlenmeyer flasks. Later, the fermentation procedures were followed as in Section 2.3 with fermentation time of 15 h. For analysis, the highest ethanol produced was used for comparison between the medium.

Table 1: Dilution of glucose stock (40 %, w/v) with seaweed liquid for fermentation medium

Medium	Seaweed liquid (mL)	Glucose stock solution (40 %, w/v) (mL)
0.4 g/ml seaweed liquid with 2 % (w/v) glucose	71.258	3.742
0.5 g/ml seaweed liquid with 2 % (w/v) glucose	71.261	3.739
0.6 g/ml seaweed liquid with 2 % (w/v) glucose	71.266	3.734
0.7 g/ml seaweed liquid with 2 % (w/v) glucose	71.269	3.731

2.5 Analytical analysis

2.5.1 DNS method

3 mL of supernatant from fermentation samples was analysed using DNS method (Zakaria et al., 2014).

2.5.2 Ethanol quantification by Gas Chromatography (GC)

Ethanol from all fermentation samples were quantified by Gas Chromatography (GC-2010 Plus Series, Shimadzu Corp., Tokyo, Japan) which equipped with BP20-capillary column measuring 30 m × 0.25 mm ID × 0.25 µm film thickness (SGE Analytical Science, Australia) and flame ionization detector. The operating conditions were as follows: detector temperature 250.0 °C, injector temperature 220 °C and oven temperature 80 °C for 3.5 min with 1 µL of injection sample volume. Standard curve for ethanol was prepared by diluting 99.99 % ethanol (HPLC grade) into desired concentrations and area for respective concentration was obtained. Ethanol peak from the fermentation samples were determined by comparing retention time of the sample with ethanol standard peak.

2.6 Calculation of ethanol yield

Ethanol yield which has been produced during fermentation were calculated as follows:

$$Y_{P/S} = \frac{Ethanol\ concentration}{Substrate\ concentration} \times 100\% \quad (1)$$

$Y_{P/S}$ refers to ethanol yield and the theoretical maximum ethanol yield is 51 %. Meanwhile, the ethanol concentration refers to maximum ethanol achieved during fermentation (M) and the substrate concentration may refer to total reducing sugar, galactose or glucose concentration at onset fermentation (M).

3. Result and discussion

3.1 Seaweed liquid potential as the supplement in fermentation media

In order to observe the potential of seaweed liquid as the fermentation supplement nutrient, the seaweed liquid was used in the fermentation medium with the addition of glucose or galactose as the carbon source at various concentrations. Then, the fermentation was performed by *S. cerevisiae* and the ethanol produced was collected and analyzed by GC. Figure 1 and 2 show the result of ethanol production from glucose and galactose fermentation with seaweed liquid as fermentation supplement nutrient. From Figure 1, there were six mediums with different concentration of glucose has been used for producing ethanol by using *S. cerevisiae*. No other nutrients such as yeast extract, peptone or $MgSO_4$ were added to any of the medium to promote *S. cerevisiae* growth. The supported nutrients were expected to be supplied by the nutrient contain in seaweed liquid. Hence, in all medium, except 2 % (w/v) glucose and YPD medium, were prepared by dissolving specific amount of glucose with seaweed liquid.

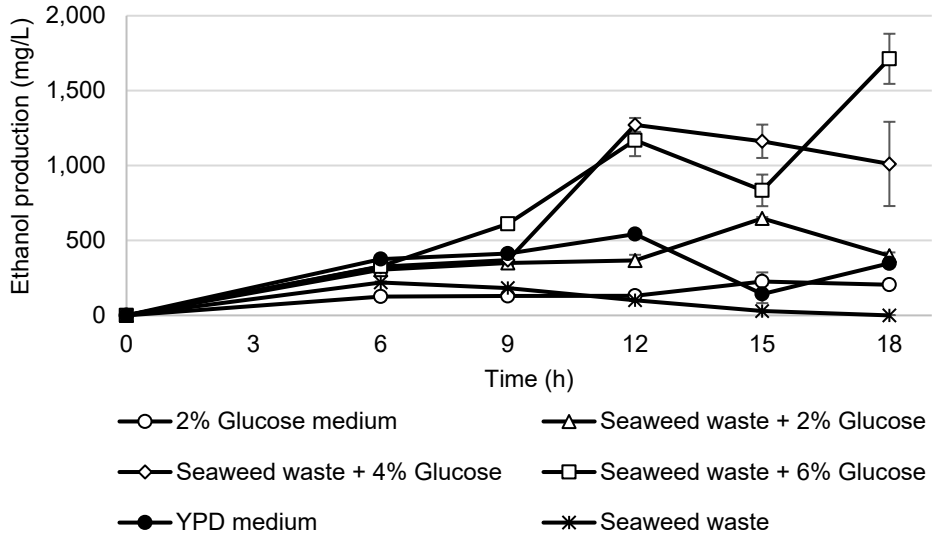

Figure 1: Ethanol production from glucose fermentation with seaweed liquid as fermentation supplement nutrient

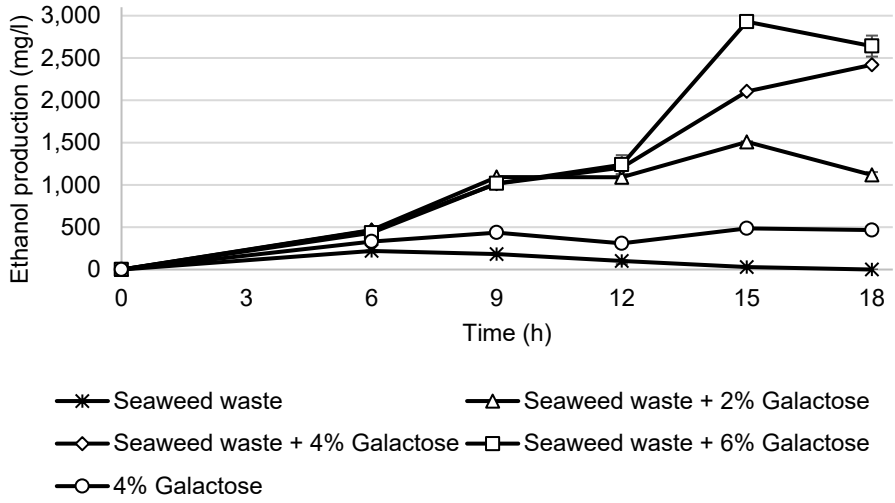

Figure 2: Ethanol production from galactose fermentation with seaweed liquid as fermentation supplement nutrient

From the result obtained as displayed in Figure 1, there were significant improvements of ethanol concentration being produced by *S. cerevisiae* when the medium used seaweed liquid as the supplement. Glucose medium (2%, w/v) and seaweed liquid medium gave the lowest ethanol concentration which is 226.10 mg/L and 219.55 mg/L when compared to other medium. Meanwhile, 6% (w/v) glucose with addition of seaweed liquid gave the highest production of ethanol which is 1712.51 mg/L and followed by 4 % (w/v) glucose with seaweed liquid, 2 % (w/v) glucose with seaweed liquid and YPD medium.

Typically, in industry or research laboratory, YPD medium has been widely used for *S. cerevisiae* growth medium as it suits the yeasts' growth very well (Chen et al., 2021). The macronutrient in YPD medium was obtained from dextrose content (carbon source) and micronutrients was supplied by yeast extract and peptone. The yeast extract in the YPD medium aids in the supply of B - complex vitamins and amino acids, whereas peptone serves as the source of nitrogen, vitamins and minerals required for yeast growth (Liu et al., 2021). The potential of enhancing ethanol production by yeast in 2 % (w/v) glucose with seaweed liquid as the medium was compared with YPD medium that consisted of 2 % (w/v) of glucose with the addition of 1% (w/v) yeast extract and 2% (w/v) peptone. Positive result has been obtained as 2 % (w/v) glucose with seaweed liquid was capable to enhance ethanol production by *S. cerevisiae* up to 647.48 mg/L when compared to YPD medium with 542. 39 mg/L. Economically, this finding is very valuable because YPD or its components (yeast extract and peptone) is very expensive and imported from outside source. Besides, the application of seaweed liquid is not limited in replacing these nutrients in YPD medium, but also could be applied in other medium.

In order to make a comprehensible comparison on ability to supplement ethanol fermentation by yeast extract, peptone and seaweed liquid, Table 2 was constructed based on 2 % (w/v) glucose as the main carbon source. From Table 2, it can be seen that all medium containing 2 % (w/v) of glucose as the main carbon source and only the supplementation of nutrients was varied for ethanol fermentation. Hence, the effectiveness in producing ethanol by these three medium were mainly dependent on the suitability of supplemented nutrients type

Table 2: The composition of different medium and ethanol yield

Composition	Unit	Glucose medium	YPD medium	Glucose with seaweed liquid medium
Glucose	%, (w/v)	2	2	2
Peptone	%, (w/v)	-	2	-
Yeast extract	%, (w/v)	-	1	-
Seaweed liquid	g/mL	-	-	0.36
Ethanol production	mg/L	226.10	542. 39	647.48
Yield	%	4.5	10.7	12.8

From the table, it can be concluded that medium with supplement from seaweed liquid have enhanced the ethanol yield to 12.8 %, while glucose medium only producing 4.5 % and 10.7 % by YPD medium. Thus, it was

obviously showed the potential of seaweed liquid in supplementing ethanol fermentation has surpass the potential of yeast extract and peptone.

Similar result was found when galactose was used instead of glucose (Figure 2). The same potential which previously discussed for glucose was achieved for galactose. Interestingly, when comparing the ethanol concentration being produced by 2 % (w/v) glucose with seaweed liquid and 2 % (w/v) galactose with seaweed liquid, the ethanol production by using galactose as carbon source was higher than glucose which was 1,508.27 mg/L and 647.48 mg/L. The identical pattern could be detected when comparing galactose and glucose at the same percentage of sugar.

These patterns suggest that when using galactose or glucose with addition of seaweed liquid, *S. cerevisiae* was more comfortable to produce ethanol in galactose by giving high ethanol concentration. These phenomena maybe caused by the nutrients that exist in the waste may contain some protein that can start the Leloir pathway for galactose in *S. cerevisiae* without having repression of enzyme by glucose. This suggestion was based on Brink et al. (2009), who claimed that, the enzymes involved in galactose utilization were not synthesized during a sudden shift from glucose to galactose in media as the enzyme was exist throughout the glucose-limited cultivations. However, the absence of Leloir proteins was the cause of the failure to induce a functional Leloir pathway for galactose and not by the absence of the corresponding transcripts of enzyme.

3.2 Optimum concentration of seaweed liquid as supplement nutrients for ethanol production

After the potential of seaweed liquid as supplement in fermentation has been proved, later, several concentrations of seaweed liquid have been used in supplementing 2 % (w/v) glucose medium for ethanol fermentation with the purpose to get the optimum concentration of this liquid to enhance ethanol production. Figure 3 shows the ethanol production by these medium

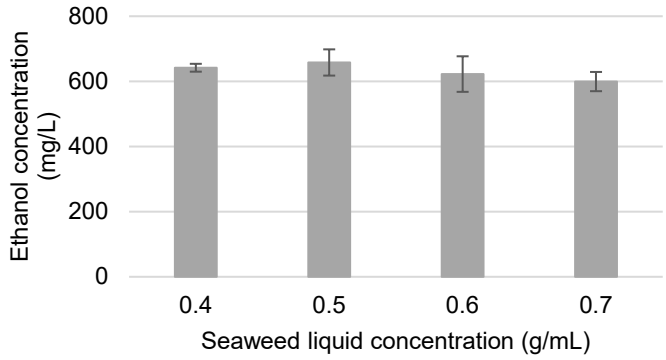

Figure 3: Ethanol production from 2 % (w/v) glucose fermentation with different concentration of seaweed liquid as fermentation supplement nutrient

From Figure 3, apparently ethanol fermentation supplemented by 0.5 g/mL of seaweed liquid gave the highest ethanol production which was 658.04 mg/L when compared to the values obtained from supplementation via 0.4 g/mL, 0.6 g/mL and 0.7 g/mL of seaweed liquid which were 641.86 mg/L, 622.44 mg/L and 599.42 mg/L. However, the results only showed slight changes in ethanol production when changed the concentration of seaweed. This is due to only small interval between the concentrations. Larger interval between the concentrations may show more impactful changes in ethanol production Initially, there was an increment of ethanol production when the concentration of seaweed liquid was increased from 0.4 g/mL to 0.5 g/mL. The increment was due to addition of minerals exist in the medium contributed by concentrated seaweed liquid. However, when the concentration of seaweed liquid exceeds 0.5 g/mL, the ethanol production started to decrease. This situation can be seen during concentration of 0.6 g/mL and 0.7 g/mL of seaweed liquid when ethanol production gradually decreased with the increased of the waste concentration. The reason for this situation happens may be caused by high amount of nutrient, minerals and heavy metals in the medium supplied by seaweed liquid, which have retarded the growth of *S. cerevisiae*. As reported by Hoda et al. (2010), excess amount of minerals such as nitrogen and ammonium ions may impede the growth rate of *S.cerevisiae*. By impeding the yeast growth, it has caused an inhibitory effect toward fermentation and subsequently reduced the ethanol production. In addition, as being reports by Alamsjah (2013), about 100 ppm of zinc (Zn) was contained in the seaweed and it is presumable that this amount might be released into the seaweed liquid. The effect of Zn toward *S.cerevisiae* growth has been reported by Shariatmadari et al. (2012). They claim that when the concentration of Zn ions taken up by the yeast was excessive, it would give harmful effect toward *S. cerevisiae*

biomass due to toxic nature of Zn. The toxicity of Zn would affect the biological inactivation of cells and loss of viability. Nevertheless, the effect of Zn in low concentration for stimulating *S. cerevisiae* growth could not be denied. As they reported that increment from 0 to 10 mg/L $ZnSO_4$ has increase the growth of *S. cerevisiae*.

4. Conclusions

Seaweed liquid can be the best candidate in replacing the application of yeast extract and peptone in growth medium especially YPD medium which was more cost effective and easy to prepared. However, the concentration of this waste must not exceed 0.5 g/ml as it would inhibit the growth of *S. cerevisiae* and indirectly reduce the concentration of ethanol being produced.

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