Microalgae cultivation in wastewater effluent from tilapia culture pond for enhanced bioethanol production
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ABSTRACT
The large number of wastewaters are generated because of the various production processes. Vegetable and fish processing can be considered an important industry for wastewater generation. The essential method for completing this waste is to digest the organic matter using anaerobic digestion followed by aerobic wastewater treatment processes; however, wastewater from tilapia culture pond retains considerable quantities of inorganic substances, particularly nutrients like nitrogen and phosphorus. The optimal conditions for cultivating Chlorella vulgaris from wastewater treatment effluent from tilapia culture pond were investigated in this study. The appropriate conditions were found to be 10% initial stock suspension, 20 cm depth, and 12 days of culture conditions. C. vulgaris had an optical density of 0.649, a cell density of $17.68 \times 10^5\text{ cells/mL}$, and biomass of $0.376 \pm 94.21\text{ mg/L}$ after cultivation. Discharged wastewater from the fishpond was utilized for the improved growth of microalgae and obtained biomass was used for bioethanol production. This study verified that fishpond wastewater is the best source of nutrients for algal mass production and biofuel applications.

Key words | biomass, fermentation, microalgae, pond effluent

HIGHLIGHTS
- Vegetable and fish processing industries are contributors to wastewater generation.
- Wastewater from tilapia culture pond retains inorganic substances.
- The optimal conditions for cultivating Chlorella vulgaris from wastewater treatment effluent.
- Discharged wastewater was utilized for the growth of microalgae.
- This study verified fishpond wastewater is the best source of nutrient for algal bioethanol production.

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doi: 10.2166/wst.2021.194
INTRODUCTION

Vegetable and fish processing can be considered an essential industry for wastewater generation throughout the country, although wastewater significantly occurs as a pollutant from these production processes. The most important alternative to complete this waste is to digest its organic matter using anaerobic digestion followed by aerobic wastewater treatment processes. However, wastewater from this system still contains remaining inorganic substances at a relatively high amount, particularly nutrients such as nitrogen and phosphorus (Bhuyar et al. 2021). Wastewater rich in nitrogen is the best source for the cultivation of microalgae. This is an important reason for impurities and eutrophication when it contacts natural water resources (Tsai et al. 2012; Ramaraj et al. 2014, 2015); on the other hand, microalgae can be used for biofuel production and nutrient removal is a promising concept for integrated biomass generation and subsequent wastewater treatment. Therefore, academics, government agencies, and industrial companies are all interested in microalgae because of their potential to aid in removing nutrients while also contributing to the production of valuable biomass (Tsai et al. 2017; Wannapokin et al. 2018).

Nevertheless, these waste substances can be developed to become important food sources for algae with high economic significance (Saengsawang et al. 2020). Previous researches demonstrated that several kinds of algae are used in culture to increase its amount in wastewater treatments, such as *Spirulina* sp., *Chlorella* sp., *Scenedesmus* sp., etc. (Gantar et al. 1991; Anaga & Abu 1996; Phang et al. 2000; Ramaraj & Dussadee 2015). Algae can be used as the best source of food for aquatic fishes. Aside from improving water quality, these algae can be developed into animal feed, significantly as a feed mixture supplement for fish feed to improve yield quality (Bai et al. 2001; Ajiboye et al. 2012). Higher industrialization of fossil fuels has enabled us to decrease our dependency on non-renewable fossil fuels while simultaneously increasing carbon dioxide emissions (Khunchit et al. 2020; Nguyen et al. 2020). These technologies help the environment and the economy, which means that microalgae can be used to a greater extent. Microalgae processing was assumed to be viable for producing goods and services, and it was determined that it could be done.

In this research, the microalgae *Chlorella vulgaris* isolated from the vegetable and fish processing industrial effluent was investigated. The novelty of this study aimed to reutilize fish water effluent for the mass cultivation of microalgae for the ultimate dual purpose of fish feed manufacture and biofuel generation. The study focused on optimizing the suitable conditions for algae cultivation and using the cultivated algae as a fish feed additive for tilapia (*Oreochromis niloticus* Linn.). The use of cultured algae grown from vegetable and fish processing effluents for fish feed additive production could reduce the capital cost for fish rearing and, at the same, serve as a guideline in treating wastewater effluents with the highest efficiency. Aside from these, the algae can be used in the future in other agricultural product factories with similar effluent characteristics.

MATERIALS AND METHODS

Wastewater effluent sample

The effluent utilized in this research was collected from vegetables, fruits and fish canning industrial wastewater treated
by anaerobic digestion followed by the aerated lagoon. The effluent characteristics were BOD 25–35 mg/L, COD 65–80 mg/L and pH 6.5–7.2.

Isolation, identification, and purification of algae

A single green algae cell was isolated by spreading it onto a petri plate containing medium and incubating it for two weeks at room temperature with 24 h of light. The strain was identified by streaking agar plate techniques with Jaworski’s medium under atmospheric controlled conditions and was isolated as a bacteria-free alga. The sample was examined on a species-by-species basis. The C. vulgaris was eventually separated from the other algae species by repeated selection and subculturing of the least contaminated cultures. The strains grown on plated media were then transferred to liquid media for stock suspension culture.

Mass cultivation of algae

The stock suspension of algae was inoculated in 500 mL volumetric flask containing wastewater effluent. Four treatments with three replications from algae initial concentration percentages of 2, 5, 10 and 20% were evaluated. The culture flasks were continuously aerated using an electric aerator. Within the span of 16 days, the cellular concentration and growth were determined by cell counting (Hemocytometer cell count) and optical density measurement at 560 nm (Hach instrument, Model DR2000) for every 2 days. The suitable initial algae concentration was cultivated in 200 L cement pond with 100 L working area used the effluent. The three different depth levels (20, 30 and 40 cm) were evaluated. In a period of 15 days, the cellular concentration and cell growth were determined every 3 days. For mass culture, the algae’s batch culture was conducted using the suitable initial algae concentration, depth and retention time based on a previous study in a 200 L cement pond with 100 L working area (Gantar et al. 1991). The algae biomass was harvested, dried and analyzed for the nutrient value according to Association of Analytical Chemists (AOAC) method (AOAC 1984).

Feed supplement for Tilapia (Oreochromis niloticus Linn.)

The mass cultivated algae were dried and ground into powder, mixed with the commercial fish pellet and later coated with fish oil. Samples were dried in the open air and were later kept in the bag. Tilapia (Oreochromis niloticus Linn.) was cultivated in cages for 3 months in a vegetable and fish canning oxidation pond. The study was divided into two treatments with three replications each. Treatment 1 consisted of commercial fish pellet, while treatment 2 comprised commercial fish pellets mixed with 3% dried algae. Feed was provided twice a day between 09:00–10:00 am and 3:00–4:00 pm, respectively. The quantity of feed was adjusted based on the monthly weight of fish. The monthly weights were recorded, and the feed supplied used to compute the growth and nutrient utilization parameters within the following equations:

Specific growth rate (%/day)

\[
\text{Specific growth rate} = \frac{\ln W2 - \ln W1}{\text{no. of days during the experiment}} \times 100
\]

where:

\[W2 = \text{final weight of fish,} \]
\[W1 = \text{initial weight of fish.}\]

Survival(%) = \frac{F1}{F2} \times 100

where:

\[F1 = \text{number of fish at the beginning of the experiment,} \]
\[F2 = \text{number of fish at the end of the experiment.}\]

Feed conversion ratio (unit) = \frac{\text{feed intake(g)}}{\text{fish weight gain(g)}}

Weight gain (g) = W2 - W1

where:

\[W2 = \text{final weight of fish,} \]
\[W1 = \text{initial weight of fish.}\]

Water quality during fish cultivation

Water quality was examined during the cultivation by floating baskets during the start of the experiment and after every 14 days until the end of the experiment. The parameters included pH, temperature, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS) (APHA 1998), nitrate, ammonia and phosphate by spectrophotometer (Hach instrument, Model DR 2500). Four heavy metals, cadmium (Cd), copper (Cu), zinc (Zn) and mercury (Hg) were also measured (APHA 1998) at the end of the experiment.
Statistical analysis

The data from the measurement were statistically analyzed by variance analysis (ANOVA). Scheffe’s method was used for the comparison of treatment mean at $P < 0.05$.

RESULTS AND DISCUSSION

Suitable initial concentration for algae

The microalgae that were isolated were described by referring to authors Nithin et al. (2020). Microalgae observed under the fluorescent microscope, the microalgae (Figure 1) have the same characteristics as Chlorella sp. According to the observation, the microalgae were green in color, indicating that they belong to the Chlorophyta division. Chlorella has a circular or oval shape with a diameter ranging from 2 to 15 m. Chlorella is a genus of Trebouxiophyceae, order Chlorellales, family Oocystaceae, and class Trebouxiophyceae. This species is typically found in a cluster or as a single individual. The microalgae C. vulgaris was isolated from the effluent (Figure 1) and used for mass cultivation. The initial stock culture of the selected algae species, C. vulgaris was cultivated in 500 mL flasks of effluent from the vegetable and fish processing industry, each containing final concentrations of 2, 5, 10 and 20% cell suspension, respectively.

The study results showed that the algae stably maintained its growth, as evidenced by increasing optical density (OD) value and the number of cells (Figure 2(a) and 2(b)). For the startup, the 20% initial stock solution showed the highest growth because of algae’s opportunity to adapt and survive much better than those of lower initial concentrations. OD value was highest on the 10th day of the experiment at 0.32, with numbers of cell at $12.43 \times 10^5$ cells/mL. Later, however, the algae multiplied in large amounts leading to death and precipitated to the bottom as sediment. This was observed in the green color of algae biomass at the bottom of the pond because of the effluent’s limited volume of feed substances. When the 10% initial cell concentration of algae was considered, steady growth was slowly maintained until the highest cell density on the 16th day of the experimental period ($16.08 \times 10^5$ cells/mL) with OD value at 0.42 was achieved. Simultaneously, when the initial concentration was 2% and 5%, the highest cell populations were reached similarly on the 16th day ($13.83 \times 10^5$ and $13.39 \times 10^5$ cells/mL, respectively) with OD values of 0.35 and 0.30, respectively. The obtained results confirm that 10% initial cell concentration is the promising inoculum concentration for the enhanced microalgae growth. The inoculum

![Figure 1](image1.png)

**Figure 1** Microscopic identification of C. vulgaris at 100× magnification.

![Figure 2](image2.png)

**Figure 2** (a) The growth of C. vulgaris cultivated at various initial concentrations by OD$_{560}$nm. (b) The growth of C. vulgaris cultivated at various initial % concentrations plotted by cell densities.
concentration is an important criterion in microalgae’s growth and development (Bhuyar et al. 2019).

A suitable initial optimum concentration is considered necessary for the cultivation of algae. During the initial period, if algae are too less concentrated, it would cause algae to adapt itself intensively. A huge number cause algae to be unable to survive; only a few would continue to grow because of breaking colonies caused by photo-oxidation (Benchokroun et al. 2003; Baroli et al. 2004). However, if algae started at too high a concentration, the yield would not be good because they would obstruct light from each other and there would be reduced photosynthesis (Gitelson et al. 1996). Simultaneously, it would also increase the investment cost of production and may lead to economic imbalance.

**Suitable depth level for algae growth**

The initial 10% *C. vulgaris* stock suspension was cultivated in 200 L cement pond with 100 L working area with three different depth levels (20, 30 and 40 cm); industry effluent was evaluated. In 15 days, the cellular concentration growths of *C. vulgaris* are illustrated in Figure 3(a) and 3(b).

Based on the results (Figure 3(a) and 3(b)), it was found that algae cultivated in 20 cm depth pond gave the best cells growth of $18.14 \times 10^5$ cells/mL and OD value of 0.546. During 0–3 days of the beginning, the algae growth was slow and slightly increasing because, at this time, they were adapting themselves to the new environmental conditions and were not yet starting to increase their number. After the initial adaptation, they started to increase their number quickly during the 3–12 days. Then the algae grew slowly again until they become stabilized. As observed during the 15th day, the highest number of cells was $18.14 \times 10^5$ cells/mL and OD value of 0.546. However, the best for harvesting period were collected on the 12th day when the algae were completely mature and only a few cells were found dead besides having a lesser amount of Chlorellin (Shields & Durrell 1964).

*Chlorella* sp., when aged, are usually released to inhibit the growth of other living matters, thus referred to as ‘Chlorellin’ (Pratt et al. 1945; Bhuyar et al. 2020), considered as an element between fatty acid and hydrocarbon (Spoehr & Milner 1973) and having allelopathic activity, a property that allows it to inhibit the growth of other living constituents together with the surrounding bacteria and algae. Aside from this, it was also found that if Chlorellin has lesser concentration, algae could continue to grow. However, if Chlorellin is present in a concentration higher than 6.5 mg/L, it would influence its own growth (DellaGreca et al. 2010). Chlorellin extracted from *Chlorella* sp., being more aged, was still able to obstruct the photosynthesis activity (Mandalam & Palsson 1995; Liu et al. 2017) harvesting; it must be collected before sedimentation or before the stationary phase.

When the depth levels at 30 and 40 cm were considered, it was found that algae growth was reduced compared with the 20 cm depth. The reason might be that *Chlorella* sp. is an alga of smaller size and able to spread out widely in the water, which affected photosynthesis. At deeper depths, light cannot effectively penetrate from the surface of the water affecting suboptimal photosynthesis, which decreases the survival rate of algae in the pond (Slegers et al. 2013). This was observed in the 40 cm depth where algae did not grow well and the period of adaptation or lag phase was much longer. However, the cultivation of *Chlorella* sp. in wastewater from the vegetable and fish processing industry was not so efficient with *Chlorella* sp. Commonly, when algae were cultivated in wastewater, they were widely accepted to feed some food substances as a supplement to...
the algae. Nevertheless, the supplementation of food substances would also increase the wastewater substances affecting the industry’s quality of effluence.

Algae productivity and mass cultivation

In this study, algae were cultivated by the batch system in a cement pond with a volume of 200 L at a suitable depth of 20 cm. Algae seed concentration from 10% effluent was harvested after 12 days during the log phase when algae grew well and had less mortality, thus having good quality. Results of the study showed that algae had an OD value of 0.649, cell density of $17.68 \times 10^5$ cells/mL, dry weight of $0.376 \pm 94.21$ g/L and this yield was not significantly different when compared with other algae cultivation methods grown in an effluent from fertilizer production industry where dry cell weight of algae was 0.541 g/L. In contrast, in Bold’s Basal Medium (BBM), dry weight was 0.629 g/L (Toyub et al. 2007) and in the Trebon system, the production rate for C. vulgaris was 10 g/L (Doucha et al. 2005). Cultivated algae had nutritional properties composed of protein at 45.6%, fiber at 8.5%, ashes at 17.0%, fat at 15.2% and carbohydrate at 10.6% of dry weight.

Bioethanol production

Because of its energy security and environmental protection over fossil fuels, bioethanol has emerged as one of the most advantageous fuels due to the decline of fossil fuels (Vu et al. 2018; Manmai et al. 2020a; Sophanodorn et al. 2020). It is an oxygenated, environmentally friendly method because it contains 34.7% oxygen, which is absent in gasoline. Since bioethanol contains oxygen, it has a 15% higher combustion efficiency than gasoline, resulting in lower particulate nitrogen oxide emissions (Manmai et al. 2020b). Other hazardous gases released by gasoline, such as sulfur oxide and carbon monoxide, can be minimized by combining ethanol with gasoline (Nithin et al. 2020). These toxic gases lead to acid rain or enter the water supply, contaminating potable water supplies and posing a health risk. Fermentation is the metabolic mechanism by which microorganisms turn soluble sugars into alcohol. Some bacteria and yeast can metabolize carbohydrates such as monosaccharides and disaccharides in the absence of oxygen, produce ethanol, and release carbon dioxide (Khammee et al. 2020). Figure 4 shows the effects of the fermentation by yeast. Three days of chemical pretreatment by 1% to 3% NaOH resulted in total sugar concentrations of 64.242 g/L, 84.253 g/L, and 94.581 g/L, respectively. After hydrolysis, reduced sugar concentrations for 1 to 3 days are 82.064 g/L, 86.312 g/L, and 94.064 g/L, respectively. During the 96-h fermentation, however, ethanol concentrations were highest at 33.213 g/L.

Fish growth performance on algae feed supplement

The study on fish growth using the cultivated C. vulgaris as feed supplement was divided into two treatments. In treatment 1, fish were fed with commercial feed while in treatment 2, fish were fed with commercial fish feed mixed

![Figure 4](http://iwaponline.com/wst/article-pdf/doi/10.2166/wst.2021.194/898633/wst2021194.pdf)
with 3% algae. Tilapias (*Oreochromis niloticus* Linn.) were grown in cages in an oxidation pond for 3 months. The results observed are shown in Table 1.

Obtained results revealed that fish fed with feed mixed with 3% *C. vulgaris* dried algae had a significant difference than the fish fed with commercial feed pellet. The algae supplemented fish shows survival rate at 95% confidence (*P* < 0.05). There was no significant difference between treatments 1 and 2 in specific growth rates, feed conversion rate and weight gain. The FCR of both treatments was very low because it took less feed to produce 1 kg of fish. Both fish diet formulas were not the only kind of feed for fish consumed because of the fish cases in the oxidation pond that still had high inorganic substrates, so plankton could grow. The plankton became a good natural food for the fish, so it was possible to use less feed to produce a kilogram of fish. However, using algae as a protein supplement for fish growing with an estimated amount to be used depending on what type of fish, such as *Paralichthys olivaceus*, which grew best when fed with 2% *C. vulgaris* powder as a feed supplement (Ajiboye et al. 2012), while with *Sebastes schlegeli*, best growth was achieved when fed with *C. vulgaris* powder at 0.5% of fish feed (*CCAP 2007*).

### Water quality during fish cultivation

After 3 months of rearing tilapia in cages in oxidation ponds, water quality analysis was shown in Table 2. Meanwhile, zinc was the heavy metal detected at a very low level of 0.01 mg/L. The water quality analysis revealed that their amounts were below the standards for inland aquaculture and industrial water quality effluent (Zhang et al. 2019; Bhuyar et al. 2020).

### CONCLUSION

In this study, *C. vulgaris* isolated from the vegetable and fish processing industry effluent was developed to become a feed supplement. The suitable situations for cultivation were initial 10% algae stock suspension, depth of 20 cm and 12 days of cultivation. Harvesting of algae yield showed that OD value was 0.649, cell density at about 17.68 × 10^3 cells/mL and dry weight of 0.376 ± 94.21 g/L. Incorporating the cultivated algae at 3% in fish pellet as a feed supplement, the results showed that fishes fed with algae supplements had better survival rates than those without supplementation. Meanwhile, water qualities were acceptable for aquaculture during the experiment and were still within the effluent standard norms for aquaculture and industrial effluent standard. The results of fermentation have revealed a consistent final ethanol concentration. After 96 h of fermentation, yeast has the highest concentration of ethanol (33.213 g/L). As a result, tilapia fish pond cultivated algae (*C. vulgaris*) can be considered a

| Table 1 | Growth performance of Tilapia in experiment pond using *C. vulgaris* as feed supplement during the 60 days experiment period |
|---------|---------------------------------------------------------------|
| Parameters | Treatment 1 (commercial fish pellet) | Treatment 2 (commercial fish pellet + 3% algae) |
| Specific growth rate (%/day) | 2.16 ± 0.006ns | 2.37 ± 0.132ns |
| Survival rate (%) | 61.7 ± 9.5ns | 76.3 ± 5.5ns |
| Feed conversion rate (FCR) | 0.45ns | 0.40ns |
| Weight gain (g) | 124.3 ± 5.19ns | 150 ± 7.58ns |

*Note*: Asterisks (*) show significant statistical differences (*P* < 0.05); ns – no significant statistical differences.

| Table 2 | Assessment of water quality during tilapia fish cultivation after three months |
|---------|---------------------------------------------------------------|
| Water Quality Index | Week 0 | Week 2 | Week 4 | Week 6 | Week 8 | Week 10 | Week 12 | Ave ± SD |
| Temp (°C) | 23.6 | 23.4 | 24.5 | 25.6 | 24.2 | 24.6 | 24.4 | 24.3 ± 0.7 |
| TS (mg/L) | 67 | 64 | 79 | 76 | 56 | 65 | 64 | 67.3 ± 7.8 |
| pH | 7.5 | 7.2 | 6.9 | 7.6 | 7.5 | 6.9 | 7.4 | 7.3 ± 0.29 |
| DO (mg/L) | 5.5 | 5.2 | 5.5 | 6.5 | 6.5 | 5.8 | 5.4 | 5.8 ± 0.5 |
| COD (mg/L) | 68 | 80 | 76 | 65 | 82 | 75 | 79 | 75.0 ± 6.3 |
| BOD (mg/L) | 16 | 18 | 19 | 15 | 14 | 19 | 20 | 17.3 ± 2.3 |
| NO₃-N (mg/L) | 17 | 19 | 14 | 25 | 16 | 14 | 19 | 17.7 ± 3.8 |
| NH₃-N (mg/L) | 0.14 | 0.16 | 0.23 | 0.15 | 0.30 | 0.24 | 0.23 | 0.2 ± 0.06 |
| PO₄-P (mg/L) | 0.11 | 0.32 | 0.16 | 0.22 | 0.30 | 0.23 | 0.18 | 0.22 ± 0.07 |
potential feedstock for bioethanol production that is both promising and effective.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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