

Astaxanthin production from sewage of traditional Thai rice vermicelli

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Abstract. This research aimed to investigate an optimal condition for astaxanthin production by *Phaffia rhodozyma* TISTR 5730 in two different media: synthetic YM medium and the medium added with coconut water and diluted with sewage from Thai traditional rice vermicelli plant (coconut water: sewage of 1:0, 1:1, 1:3 and 1:5 ration respectively). The basic medium formulation was composed of 10 g/L glucose, 3 g/L yeast extract, 0.1 g/L K_2HPO_4 , 0.01 g/L NaCl, 0.01 g/L $MgSO_4$ and 0.01 g/L $CaCl_2$ with initial pH 5.5. The cultures were cultivated on 200 rpm shaking bath at 50 °C for 120 hr. It was found that *P. rhodozyma* TISTR 5730 grew optimally when cultivated in a mixture of coconut water and Thai rice vermicelli sewage (ratio of 1:3), with growth of 3.23 g dry biomass/L and specific astaxanthin production of 680 µg/g dry cell respectively. When fan palm sugar was added to increase reducing sugar from 10 to 15, 20 and 25 g/L, it was demonstrated that the 15 g/L formulation produced highest both dry cell weight (9.66 g/L) and astaxanthin (810 µg/g dry cell weight). Furthermore, when 0.5, 1.0 and 1.5 g/L citric acid was added as supplement, it was found that 1.0-g/L citric acid formulation gave the best result: 10.30 g/L dried cell weight and 930 µg/g dry cell weight astaxanthin. This study provides a promising alternative method of sewage reduction and valorization of wastewater from Thai traditional rice vermicelli plant.

1. Introduction

Rice Vermicelli (a traditional rice noodle) is very famous in Thailand, Malaysia and Indonesia. Its demand has been met by established factories which exist in quite different scales ranging from household, small, medium and big scales. In Thailand, the noodles are produced from rice flour and the process uses large amount of water, thus creating a large volume of wastewater which must be treated in some ways. In normal processing condition, wastewater from this process has BOD of 3,000-5,000 mg/L and COD of 4,000-5000 mg/L approximately. Large amount of wastewater with this level of COD/BOD and low pH (approximately 5.5). gives rise to a considerably high cost in compulsive wastewater treatment. To mitigate the problem, some rice vermicelli factories turn this wastewater



into biogas. However, this option is not very attractive because the COD of the wastewater is not high enough to be cost effective considering high investment in infrastructure and equipment.

A few researchers have explored other alternatives to better utilize the wastewater including that for producing bioethanol [1,2,3]. Probably one of the better idea is to turn this wastewater into high-value products by fermentation. Many researchers have explored this approach for various type of wastewater and astaxanthin is one product that has fulfilled this potential. Astaxanthin is a pigment in the xanthophyll family of carotenoids which is considered to be one of the most valuable compounds with a wide range of applications in the food, feed, cosmetics and pharmaceutical industry [4,5,6]. Its main characteristic is anti-free radical. It helps to prevent cancer in large intestine, digestion system, urine excretion system [7]. It is very popular as a feed supplement in salmon, shrimp and crab farming to induce red color in skin and their meat. High-grade asthaxanthin is a desirable, high-value ingredient for cosmetics. Currently all astaxanthin used in Thailand is imported. Global market for astaxanthin in aqua feed, nutraceuticals, cosmetics and food & beverages is estimated at 280 metric tons valued at US\$447 million in 2014. This figure is climbing up gradually and it is predicted that in 2020 the total value of astaxanthin will exceed US\$ 1.1 billion [8]. This work investigates the potential of astaxanthin production using wastewater (or sewage) from Thai rice vermicelli factories (called TRVF wastewater). Our long-term aim is to convert considerable wastewater from this source into some high-value product such as astaxanthin in such a way that the business is more profitable while creating more environmental-friendly communities.

2. Material and methods

3.1 Inoculum preparation

Phaffia rhodozyma TISTR 5370 was obtained from Thai Institute of Science and Technology, Phatumthani, Thailand. It was maintained in agar slant containing YM in 40 % glycerol. Before carrying experiments, the yeast was activated by growing in 50 ml of liquid medium (in 250-ml flask) containing YM broth which consisted of 1% glucose, 0.5% peptone, 0.3% malt extract and 0.3% yeast extract. The culture was incubated in a shaking incubator controlled at 25 °C, 200 rpm, the cool-white-fluorescent light having intensity of 500 lux, for period of 24-48 hr. The initial pH of medium was adjusted to 5.5 by 1N HCl before being sterilized at 121 °C for 15 min. The starting inoculum was 10% of the medium content which is equivalent to $10^7 - 10^8$ cfu/ml (measured as absorbance of 0.5 nm) [9]

3.2 Wastewater handling and pre-treatment

The wastewater from traditional Thai rice vermicelli processing was collected from a factory in Kanthang district, Trang province. To ensure the uniformity as well as minimum contamination, the cooled wastewater left from the noddle-boiling step inside the boiling pan was used in all experiments. After being cooled, it was collected in plastic containers (500 L total volume) which were covered with ice during transportation from the factory to our laboratory. At the laboratory, all transported wastewater was mixed together and heated at 60 °C for 30 min. Then it was left to cool down and frozen until used in the experiments.

3.3 Media preparation

In this work, YM broth was used as the controlled medium as well as for inoculum preparation. The media used in the experiments were adapted from [9] and [10]. They (called CN media) have coconut-water-to-wastewater ratios of 1:1, 1:3 and 1:5 respectively. All formulas were supplemented with nutrients to contain 10 g/L glucose, 3 g/L yeast extract, 0.1 g/L K_2HPO_4 , 0.01 g/L NaCl, 0.01 g/L $MgSO_4$ and 0.01 g/L $CaCl_2$. The initial pH was adjusted to 5.5. This is based on the work of Hu *et al.* [15] as they found that the best optimal initial pH for cell growth was 6.0 whereas the optimal pH was 5.0 for astaxanthin accumulation. Thus the compromised initial pH would be at the neighbourhood of

5.5. Two sources of carbon were investigated, namely: fan palm sugar (the media were adjusted to contain 15, 20, and 25 g/L glucose) and citric acid (citric acid was added and adjusted to contain 0.5, 1.0, and 1.5 g/L citrate respectively). In case of citric acid addition, it is considered as a precursor for astaxanthin production rather than the main carbon source.

3.4 Fermentation

The culture was grown in 250-ml flask having 50-ml broth which was prepared as in previous section. The flasks were incubated in a 200-rpm shaking incubator equipped with 500-Lux from cool white fluorescent lamp, 25 °C for 6 days (144 hr).

3.5 Dry cell and chemical analysis

Microbial growth was measured by monitoring the turbidity (by measuring light absorbance at 660 nm wavelength) as well as by measuring dry-cell content according to Kurane *et al.* [11]. Reducing sugar analysis was in accordance with Somogyi-Nelson method [12]. Astaxanthin content was analysed by a method adapted from [13] and [14]. From previous experiences, which is confirmed by the results in this study, insignificant astaxanthin is produced and accumulated before 48 hr after starting fermentation, thus we started analysing astaxanthin after 48 hr of fermentation until day 6 (120 hr). COD, BOD, total solid (TS), total soluble solid (TSS), oil, volatile soluble solid (VSS), total Kjeldahl nitrogen (TKN) were analysed according to APHA, WCPF standard analysis for wastewater [17].

3.6 Calculation of kinetic parameters

Maximum specific growth rate μ_{max} was calculated from the initial slope of each growth curve (g dry cell mass versus time) of the experiment divided by the instant biomass. Biomass yield coefficient $Y_{x/s}$, astaxanthin yield coefficient $Y_{p/s}$, and specific astaxanthin yield $Y_{p/x}$ were calculated by the following formulas.

$$Y_{x/s} = \frac{\text{dry biomass produced in a time period}}{\text{glucose consumed in that time period}}$$

$$Y_{p/s} = \frac{\text{astaxanthin produced in a time period}}{\text{glucose consumed in that time period}}$$

$$Y_{p/x} = \frac{\text{astaxanthin produced in a time period}}{\text{dry biomass produced in that time period}}$$

All time-course data presented in this work were obtained by averaging results from triplicate experiments.

4. Result and discussion

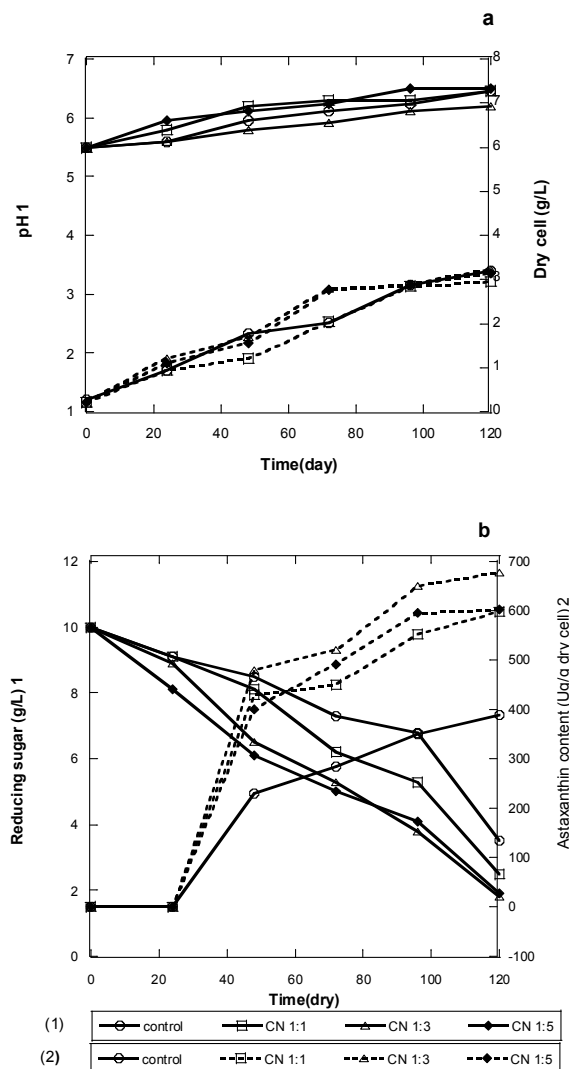
4.1 Characteristics of wastewater from processing of traditional Thai rice vermicelli

Traditionally, the process of Thai rice vermicelli started from fermenting flour-cake anaerobically for 1-2 days, then it was boiled to turn into noodle-like vermicelli while the used water became wastewater to be treated before being released into the natural waterways. The wastewater, having white color, was filtered with thin cloth to remove particulates, kept in the fridge and analysed for COD, BOD, total solid (TS), total soluble solid (TSS), oil, volatile soluble solid (VSS), total Kjeldahl nitrogen (TKN) and initial pH. The results are shown in Table 1. This results suggested that the wastewater fell within the standard for industrial wastewater which can be disposed after treatment. Furthermore, the initial pH of the wastewater is suitable for astaxanthin production [15] given all nutrients are sufficient and balanced. However, the protein and fat concentration were too low to be used directly for astaxanthin production. Thus nutrient supplement was added as elaborated in previous section.

4.2 Growth of *Phaffia rhodozyma* TISTR 5730 in coconut-water mixed with TRVF wastewater

Table 1. Characteristics of sewage from traditional Thai rice vermicelli plant

Parameters	Values
BOD (mg/L)	4,000
COD (mg/L)	5,288
TS (mg/L)	3,250
TSS (mg/L)	1,500
Oil (mg/L)	45
VSS (mg/L)	500
TKN (mg/L)	150
pH	5.5

**Figure 1** Growth and astaxanthin production of *P. rhodozyma* TISTR 5730 cultured on three different coconut-water-to-wastewater ratios of 1:1, 1:3 and 1:5 respectively: a) pH and dry cell and, b) Reducing sugar (or approximately glucose) and astaxanthin content

The experimental results from batch culturing of *Phaffia rhodozyma* TISTR 5730 in coconut water (CN) diluted with different ratio of coconut-water-to-TRVF-wastewater (1:0, 1:1, 1:3 and 1:5 respectively) are shown in Figure 1. The corresponding parameters are presented in Table 2.

Table 2. Effect of different coconut-water-to-wastewater ratios (1:1, 1:3 and 1:5) on the pH, biomass, astaxanthin content, maximum specific growth rates and yield coefficients for *P. rhodozyma* TISTR 5730 grown at 25 °C and 200 rpm under a light intensity of 500 lux for 120 h.

Coconut-water-to-wastewater ratio →	Control	CN 1:1	CN 1:3	CN 1:5
pH	6.45	6.50	6.20	6.50
Biomass (g dry cell/L)	3.19	2.95	3.23	3.15
Maximum growth rate, μ_{max} h ⁻¹	0.15	0.14	0.11	0.13
Glucose consumed after 120 h, (g glucose/L)	6.3	7.2	8.3	8.1
Biomass yield coefficient, $Y_{x/s}$ (g dry cell/g glucose)	0.51	0.41	0.39	0.39
Astaxanthin conc. (μg/L)	1,244	1,741	2,210	1,922
Specific astaxanthin yield $Y_{p/x}$ (μg/g dry cell)	390	590	680	610
Astaxanthin yield coefficient, $Y_{p/s}$ (μg/g glucose)	197	242	265	238

Starting from pH 5.5, the final pH after 120 hr was in the range of 6.2 to 6.5 and CN 1:3 had the lowest final pH of 6.2 which is closed to an optimal pH (5.0 to 6.0) for *P. rhodozyma*, particularly optimal pH for cell growth [15]. It was evident that the best coconut-water-to-TRVF-wastewater ratio was 1:3 after cultured in 250 ml flask, 200-rpm on shaker incubated at 25 °C for 120 hr. At this condition, 3.23 g/L of dry cell and 680 μg astaxanthin/g dry cell weight was achieved with small pH change (pH 5.5-6.2). It was also found that at the ratio of 1:1 and 1:5 the total cell growth was less than control experiment (basic medium without TRVF wastewater addition). However, the amount of astaxanthin concentration produced was not directly related to the amount of cell dry weight. Although the ratio of 1:1 and 1:2 gave lower growth (2.95 and 3.15 g dry cell/L respectively) than the control one (3.19 g dry cell/L), the astaxanthin produced was about double of the control. This indicated that adding TRVF wastewater into coconut-water medium induced astraxanthin production considerably. It was also noticed that the amount of astaxanthin produced followed closely the final pH, of which, in the experimental range, lower pH was associated with higher astaxanthin concentration. This is consistent with results of Sujarit et al [9,10] and Hu et al [15] who concluded that, for *P. rhodozyma*, pH 6.0 is optimal pH for cell growth whereas the optimal pH for astaxanthin production is 5.0.

4.3 Effect of sugar concentrations

To understand the effect of sugar concentration, palm sugar were added into the best medium chosen from the previous experiments (coconut juice medium mixed with TRVF wastewater at the ratio of 1:3) to adjust the initial reducing sugar concentration such that additional three levels of the concentration (15, 20 and 25 g/L respectively) were obtained. Note that one liter of this palm sugar syrup contains 28.8 g glucose, 26.0 g sucrose, 28.3 g fructose, 0.2-0.3 g protein and 0.98 g citric acid [16]. It's initial pH was 4.69. However, it should noticed that we added crystallized palm sugar not the syrup one.

Figure 2 shows the experimental results from batch culturing of *Phaffia rhodozyma* TISTR 5730 in coconut water (CN) adjusted to three glucose concentrations (15, 20 and 25 g/L respectively) by adding fan palm sugar. The corresponding parameters are presented in Table 3.

Again, the batch fermentation was carried out in 250 ml flask, 200-rpm on shaker incubated at 25 °C, white fluorescent 500-Lux light intensity, for 120 hr. It was evident that, with the sugar range of 0-25 g/L, *P. rhodozyma* TISTR 5370 grew well and consumed most of carbon source for all levels of initial sugar concentrations. As the reducing sugar increased from 10 g/L to 15 g/L, cell growth was higher and biomass yield coefficient $Y_{x/s}$ increased from 0.51 to 0.77 g dry cell weight /g sugar consumed. However, although the sugar was consumed and brought to a low level (< 3.5 g/L), biomass yield coefficient became lower as sugar concentration got higher if the initial sugar concentration was higher than 15 g/L. This indicated that, in the current physico-chemical conditions, the yeast appeared to have a growth limit (which is in the neighbourhood of 10 g/L) and the increase in sugar concentration did not help to promote growth to exceed this limit. This limit could be lifted up by increase oxygen transfer rate which can be achieved by stronger mixing [19].

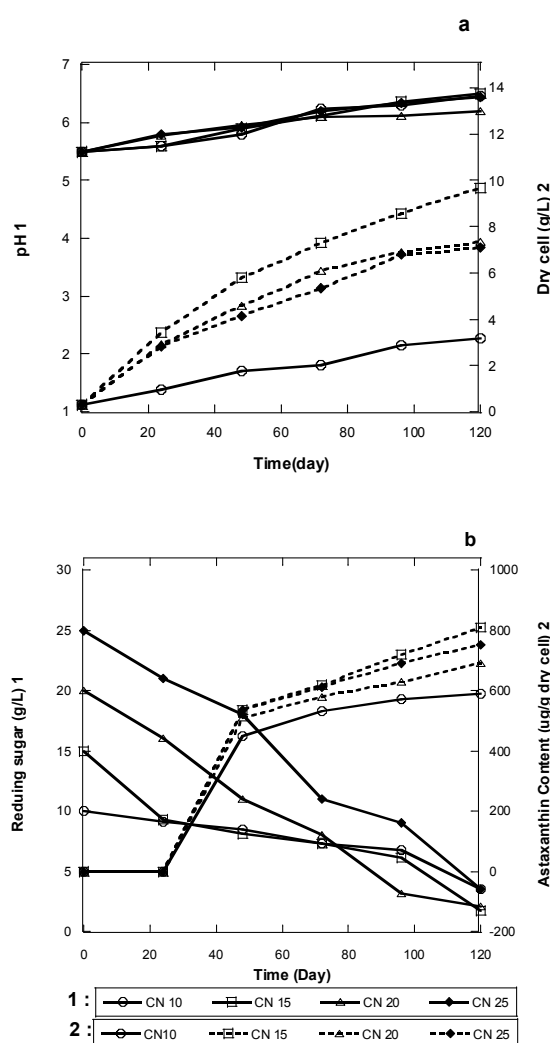


Figure 2 Growth and astaxanthin production of *P. rhodozyma* TISTR 5730 cultured on three levels of the glucose concentration - 15, 20 and 25 g/L respectively (fixed coconut-water-to-wastewater ratios of 1:3) a) pH and dry cell and, b) Reducing sugar (or approximately glucose) and astaxanthin content

Higher sugar concentration than 15 g/L tended to decrease growth rate as well as the maximum biomass attained at the stationary phase. As it is well known that astaxanthin is an intra-cellular

pigment, less cell/biomass is generally associated with less astaxanthin yield. However, astaxanthin is a secondary metabolite which is produced during the fermentation by *P. rhodozyma* in both growth-associated and non-growth associated manners. Thus in general, we can infer that, with a certain physiological state of *P. rhodozyma*, the astaxanthin concentration in the cell should be approximately constant so more biomass is associated with more astaxanthin content. However, since the physiological state of *P. rhodozyma* is varied according to physico-chemical condition of the environment as well as its internal factors, we also expected the non-growth associated astaxanthin accumulation played important role in the fermentation period.

Table 3. Effect of glucose concentration (at fixed coconut-water-to-wastewater of 1:3) on the pH, biomass, astaxanthin content, maximum specific growth rates and yield coefficients for *P. rhodozyma* TISTR 5730 grown at 25 °C and 200 rpm under a light intensity of 500 lux for 120 h.

Media with different glucose conc. →	Control 10 g/L	CN 15 g/L	CN 20 g/L	CN 25 g/L
pH	6.45	6.50	6.20	6.43
Biomass (g dry cell/L)	3.19	9.66	7.35	6.78
Maximum growth rate, μ_{max} h ⁻¹	0.15	0.17	0.11	0.18
Glucose consumed after 120 h, (g glucose/L)	6.3	12.6	17.3	21.3
Biomass yield coefficient, $Y_{x/s}$ (g dry cell/g glucose)	0.51	0.77	0.42	0.32
Astaxanthin conc. (µg/L)	1,244	7,824	3,807	4,407
Specific astaxanthin yield $Y_{p/x}$ (µg/g dry cell)	390	810	518	650
Astaxanthin yield coefficient, $Y_{p/s}$ (µg/g glucose)	197	620	220	207

Higher sugar concentration corresponds to high C/N ratio. At first look, our results may not agree with those of Liu and Wu [18] who found that carotenoid biosynthesis is promoted by high C/N ratio, low carbon and nitrogen concentrations, and slightly acidic condition when cell growth is suppressed. However, while high sugar concentration means high C/N ratio (promote astaxanthin synthesis), it also corresponding high carbon concentration (demote the synthesis). Thus, our results are actually supporting the finding of the previous work [18,20] and indicate that the glucose concentration of 15 g/L met the balance between the two opposite effects of high sugar concentration and give the best result.

4.4 Effect of citric acid as an astaxanthin-enhancing supplement

There were considerable attempts to induce the production and accumulation of astaxanthin in the culture of both *Haematococcus pluvialis* and *P. rhodozyma*. Many researchers tried to optimize astaxanthin biosynthesis by pH control strategy [15,22], fed-batch strategy [23,25,27], light type and intensity [24], nitrogen source [21] and citrate supplement [26].

Figure 3 shows the experimental results from batch culturing of *Phaffia rhodozyma* TISTR 5730 in coconut water (CN) adjusted citrate concentration to 0.5, 1.0 and 1.5 g/L (fixed glucose concentrations at 15 g/L by adding fan palm sugar). The corresponding parameters are presented in Table 4.

In our study, when 0.5, 1.0 and 1.5 %wt/volume was added to the mixture of coconut juice and TRVF wastewater (1:3 by volume), initial pH 5.5, after cultured in 250 ml flask, 200-rpm on shaker incubated at 25 °C, white fluorescent 500-Lux light intensity, for 120 hr. It was found that *P. rhodozyma* TISTR 5370 grew much better than in control and CN 1:3 if without citric-acid addition (Table 5 and Figure 4). Addition of citric acid to 1.0 g/L gave the best growth and enhanced

astaxanthin production considerably. In this case, biomass concentration, in term of dry-cell mas, reached 10.33 g/L and astaxanthin amount of 930 microgram/ g dry cell was attained. This result followed the same trend as observed by Sujarit (2009) for the same microorganisms, but grown in artificial MB medium, with 1.5 g/L citric acid addition. In her work, the referred condition gave the cell and astaxanthin concentration of 13.25 g dry cell/L and 1,051 $\mu\text{g/g}$ dry cell respectively.

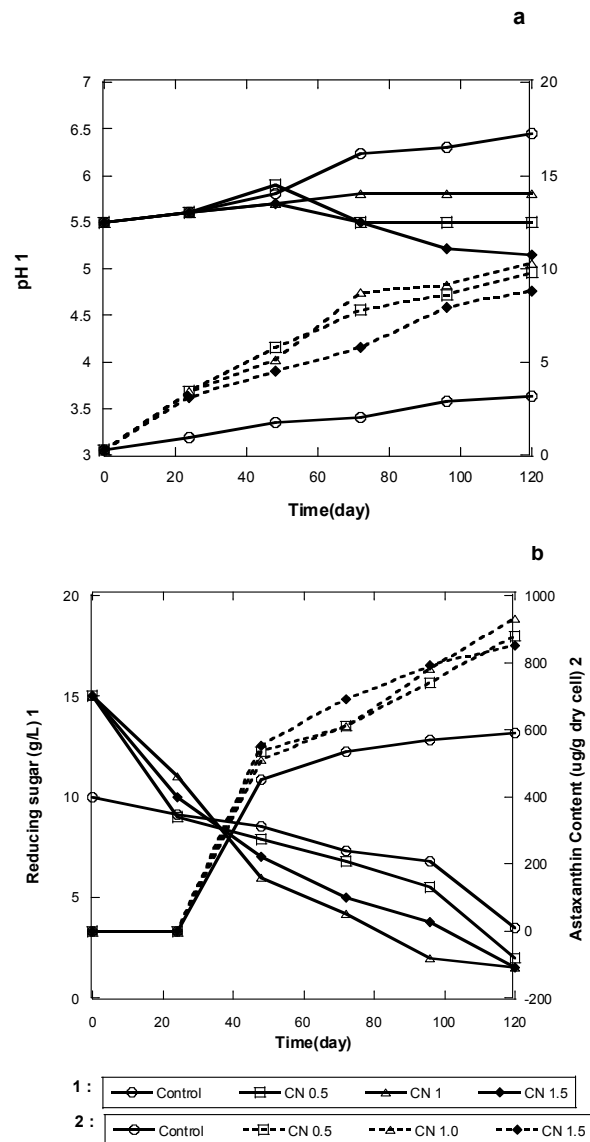


Figure 3 Growth and astaxanthin production of *P. rhodozyma* TISTR 5730 cultured on three levels of citrate concentration – 0.5, 1.0 and 1.5 g/L respectively (fixed coconut-water-to-wastewater ratios of 1:3) a) pH and dry cell and, b) Reducing sugar (or approximately glucose) and astaxanthin content

4.5 Comparison with other works

So far the best result in our work is 9,246 $\mu\text{g/L}$, 920 $\mu\text{g/g}$ dry cell and 708 $\mu\text{g/g}$ glucose for astaxanthin concentration, specific astaxanthin yield $Y_{p/x}$ and astaxanthin yield coefficient, $Y_{p/s}$ respectively. This is a typical good result for wild type strains grown in various media and physico-

chemical conditions [29,31,33,35]. However, our achieved quantities were far less than those obtained from commercial production from which well-developed microbial strains were used [28,30,32,34]. For example, Hu *et al.* [28] achieved the maximum astaxanthin concentration of 39.47 mg/L after 132 hours of fermentation, which is more than four-folds of the best result in this work. This is not surprised and our results could be improve much further if better feeding strategy and more optimized condition is used. However, suffice is to say that sewage from Thai traditional rice vermicelli plant mixed with coconut water can provide a good alternative for better waste utilization to produce astaxanthin in the future. Certainly, much more future work is needed.

Table 4. Effect of citrate concentration (at fixed coconut-water-to-wastewater of 1:3) on the pH, biomass, astaxanthin content, maximum specific growth rates and yield coefficients for *P. rhodozyma* TISTR 5730 grown at 25 °C and 200 rpm under a light intensity of 500 lux for 120 h.

Media with different citrate conc. →	Control 10 g glucose/L	CN 0.5 g/L 15 g glucose/L	CN 1.0 g/L 15 g glucose/L	CN 1.5 g/L 15 g glucose/L
pH	6.45	5.40	5.75	5.10
Biomass (g dry cell/L)	3.19	9.66	10.05	8.10
Maximum growth rate, μ_{max} h ⁻¹	0.15	0.17	0.11	0.18
Glucose consumed after 120 h, (g glucose/L)	6.3	12.6	13.0	13.0
Biomass yield coefficient, $Y_{x/s}$ (g dry cell/g glucose)	0.51	0.77	0.77	0.62
Astaxanthin conc. (μg/L)	1,882	8,404	9,246	6,925
Specific astaxanthin yield $Y_{p/x}$ (μg/g dry cell)	590	870	920	855
Astaxanthin yield coefficient, $Y_{p/s}$ (μg/g glucose)	301	670	708	530

3. Conclusion

Optimal condition for cultivating cell and producing astaxanthin in batch culture using wild-type *Phaffia rhodozyma* TISTR 5730, grown in a medium formulated by mixing coconut juices with wastewater from Rice Vermicelli factory, supplemented with 3 g/L yeast extract, 0.1 g/L K₂HPO₄, 0.01 g/L NaCl, 0.01 g/L MgSO₄, and 0.01 g/L CaCl₂, was found with respect to CW-to-TRVF ratio, sugar concentration, and concentration of citric acid supplement. For fixed initial pH of 5.5, cultivated in 200-rpm shaken flask, exposed to 500-lux fluorescent lamps, at 25 °C for 120 hr, the optimal CW-to-TRVF ratio, sugar and citrate concentration was 1:3, 15 g/L and 1.0 g/L respectively.

4. Acknowledgement

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