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Eco-friendly methods for recycling of crayfish "*Procambarus clarkii*" by-product for astaxanthin extraction and quantification

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ABSTRACT

Astaxanthin has been extracted in the current study using eco-friendly methods. Compared to the chemical method, a natural method using flaxseed oil at different time intervals, a biological method using standard fungal and bacterial probiotics (the fungal probiotics are *Saccharomyces cerevisiae* and *Candida utilis*, while the bacterial probiotics are *Lactobacillus lactis* and *Bifidobacterium lactis*), and a mixed-novel method using both flaxseed oil and the fungal and bacterial probiotics were approached. The natural extraction by flaxseed oil (used alone) resulted in a gradual increase in the astaxanthin concentration with the increase of the time interval. The highest concentration was observed in the accumulated method after 48 hours. For the biological method using probiotics, the concentration of the extracted astaxanthin by the fungal probiotics using *S. cerevisiae* was higher than that recorded by using *C. utilis*. On the other hand, a higher concentration was detected when using bacterial probiotics *L. lactis* compared to *B. lactis*. While in the mixed-novel method (using both flaxseed oil and probiotics), the highest concentration of astaxanthin was recorded in *L. lactis*, followed by *B. lactis*, *S. cerevisiae* and *C. utilis*, respectively, using the accumulated method after 48 hours. Remarkably, the concentration of astaxanthin in the chemical method was lower than in the biological and mixed-novel methods.

INTRODUCTION

Crustaceans contain 4 major classes, viz. branchinopods, copepods, ostracods and malacostraca, which include crayfish, lobster and mantis shrimp. They are invertebrates with a hard exoskeleton **(Hockmon, 2014)**. Notably, more than 10,000 tonnes of shellfish wastes may be accessible each year, providing enough raw material if the necessary commercial procedures for valuation processes were created **(Merzendorfer, 2011)**. The waste generated by shellfish processing industries each year is a practical challenge. There is a potential major environmental danger with around 75% of the total weight of

crustaceans (shrimp, crabs, prawns, lobster and krill) ending up as by-products due to the absence of appropriate waste treatment methods **(Kuddus & Ahmad, 2013)**. Typically, wastes of seafood are discarded at sea, burned, landfilled, or just left to rot **(Xu *et al*., 2013)**.

Approximately, 80 percent of crayfish is wasted exoskeleton **(Arbia *et al*., 2013; Peng *et al*., 2016)**. This huge amount of waste is hard to discard causing major environmental pollution. Crustacean waste management is a major problem facing food industries for the difficulty and the high cost of the recycling process. A small portion of waste is used as animal feed or fertilizer **(Linden & Stoner, 2007; Xu *et al*., 2008)**. While, the remaining portions are commonly discarded in landfills or into the sea in coastal regions **(Xuemei & Hawkins, 2002; Arvanitoyannis & Kassaveti, 2008)**. Proper disposal of such waste can be very expensive; whereas, unsuitable disposal can cause serious health issues for humans and the environment **(Hamed *et al*., 2016; Ouf *et al*., 2019)**.

The potentials of the crustacean shells in the fields of development and use are remarkable; they can be used as a source of beneficial chemicals for many commercial applications **(Yan & Chen, 2015; Chen *et al*., 2016)**. In addition, crustacean waste can be used to recover marine functional components, nutraceuticals and pharmaceuticals, providing valuable products with economic and environmental benefits targeting crustacean-processing regions **(Nguyen *et al*., 2017)**.

Crustacean waste could be the cheapest raw material for carotenoid recovery, and could later be a better and less expensive alternative to synthetic carotenoid supplements **(Dalei & Sahoo, 2015)**. The carotenoid astaxanthin, which is integrated into a macromolecular protein complex known as crustacyanin, determines the shell color of decapod crustaceans **(Chayen *et al*., 2003)**. Crayfish possess a variety of sizes, colors, life cycles, and habitats, with over 500 species worldwide. The red swamp crawfish, *Procambarus clarkii*, and the white river crawfish, *Procambarus acutus acutus* are two commercially important species **(Larry *et al*., 1990)**.

Astaxanthin, a xanthophyll carotenoid, is a secondary metabolite responsible for the red-orange color of many marine creatures and microorganisms. It is naturally produced by bacteria, microalgae and yeasts **(Davinelli *et al*., 2018; Tatas *et al*., 2020)**. It is considered one of the most powerful natural components for its commercial application in various industries comprising aquaculture, food, cosmetics and pharmaceuticals. Crayfish contain astaxanthin of a high impact on free radicals; it has the potential to resist oxidation, enhance immunity and prevent cancer **(Chen *et al*., 2020)**. Astaxanthin supplementation has been studied in different therapeutic applications and proved to have several pharmacological effects **(Guerin *et al*., 2003; Ambati *et al*., 2014)**. Astaxanthin has a wide range of biological activities; it has antioxidant, anti-aging and anti-cancer

properties, and has a positive role in immune-boosting, free radical scavenging, and human nutrition and health **(Fassett & Coombes, 2011; Fassett & Coombes, 2012)**. The proper recycling of crayfish shells would give highly important components that would enhance human health, grow the economy, and maintain a healthy environment.

One of the most promising methods for extraction of astaxanthin is using flaxseed oil (as a solvent) as an eco-friendly natural method (Pu & Sathivel, 2011; Scott, 2020). There are several biological methods that aim to extract astaxanthin through the fermentation of 2 species of lactobacillus bacteria (*Lactobacillus acidophilus* and *Lactobacillus plantarum*) **(Khanafari *et al*., 2007)**, in addition to the production of certain enzymes (Chitinase and protease) through the fermentation of *Aeromonas hydrophila,* where the two enzymes help in disrupting cells of shrimp wastes **(Cheong *et al*., 2014)**. Additionally, several studies discuss methods for biological extraction on *Haematococcus pluvialis* such as germination **(Kim *et al*., 2022)**. In this context, **Praveenkumar *et al*. (2015)** found that germination weakens the cell wall of *H. pluvialis*, enhancing astaxanthin extraction.

Many chemical methods were done for the extraction of astaxanthin from several organisms, for instance, **Sachindra *et al*. (2005)** used ethyl acetate, ethanol, methanol, ethyl methyl ketone, and acetone as polar solvents, and hexane in addition to petroleum ether as non-polar solvents. Other methods were performed using an equal ratio of acetone and ethyl acetate in the extraction of astaxanthin from wastes of shrimp **(Weerantunge & Perera, 2016)**. Moreover, **Ashgar *et al*. (2016)** compared between two chemical methods by using alkali (sodium hydroxide) and acid (hydrochloric acid) and enzymatic method (trypsin), where all the previously mentioned methods were executed using shrimp wastes. Various researches used supercritical carbon dioxide in extraction **(Radzali *et al*., 2016; Roy *et al*., 2020)**. The quantitative analysis for astaxanthin can be done using UV-spectrophotometry as in the study of **Sachindra *et al*. (2006)**.

The main aim of the study was recycling the exoskeleton of crayfish by eco-friendly methods (natural, biological and mixed-novel methods) in comparison with chemical method to prove that astaxanthin can be extracted using flaxseed oil and probiotics combined as a novel mixed-method for a high yield of extraction.

MATERIALS AND METHODS

Collection of crayfish and raw material

Crayfish (*Procambarus clarkii*) were gathered from Rosetta or Rashid, a port city of the Nile Delta, and samples were transported on ice.

Raw materials

Flaxseed oil was obtained from harraz market (Cairo, Egypt). Bacteria (*Bifidobacterium lactis* - accession no: DSM10140) and fungi (*Candida utilis* - accession

no: NRRL Y-660*)* were provided by the Microbiological Resources Centre (Cairo Mircen), Faculty of Agriculture, Ain Shams University. Furthermore, *Lactobacillus lactis* bacteria was obtained from yogurt, and *Saccharomyces cerevisiae* was obtained from baking dry yeast.

Sample processing

Frozen samples of crayfish were left till attaining room temperature then shells, legs, and carapace were isolated from the samples. All the waste products were washed with fresh water, and then the whole amount of shells was determined by weighing them before drying **(Hu *et al*., 2019)**. The shells were oven- dried (in 50ºC) for 10 hours then were left to be air- dried for another 24 hours. Thereafter, the shells were ground to a fine powder using a home blinder and sifted using a sieve to get fine powder; this powder was weighed then stored in clean containers with silica packets at room temperature.

Natural method for astaxanthin extraction using flaxseed oil

Crayfish powder (10g) was added to 20ml of flaxseed oil, and then the oil was withdrawn using a syringe immediately. The experiment was repeated, but with leaving the oil and the powder for 6, 12 and 24 hours.

The accumulative method after 48 hours

Crayfish powder (10 g) was added to 20ml of flaxseed oil, and then the oil was withdrawn using a syringe after 12 hours; another amount of oil was added to the remaining powder and withdrawn again after 24 hours, and then another amount of oil was added to the remaining powder and withdrawn after 48 hours.

Biological method for astaxanthin extraction using probiotics

Microorganisms and culture media

Two bacterial probiotics *(B. lactis* DSM10140 and *L. lactis)* were used. *B. lactis* (DSM10140) and *L. lactis* were sub-cultured on MRS agar [$MnSO_4$ 0.04 g, peptone from casein 10.0 g, meat extract 8.0 g, tween 80 1.0 g, CH_3COONa 5.0 g, D(+)glucose 20.0 g, $C_6H_{14}N_2O_7$ 2.0 g, $MgSO_4$ 0.2 g, yeast extract 4.0 g (MERCK)]. MRS Broth was mixed with 15 g/L agar to solidify, then incubated at 37°C in the presence of 5% CO_2 for 48-72 hours **(Khanafari *et al*., 2007)**. For bacterial astaxanthin extraction, MRS broth media was used with the same composition but without the addition of agar.

The fungal probiotics *C. utilis* (NRRL Y-660) and *S. cerevisiae*_Czapek-Dox agar were used. This medium contained the following (g/l): sucrose, 20 g $NaNO_3$, 2 g K_2HPO_4, 1 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.5 g $FeSO_4.7H_2O$, 0.01 g, and 15 g agar (Ali *et al*., 2015). Then it was incubated at 37ºC for 72 hours. For fungal astaxanthin extraction, Czapek-Dox broth media was used with the same composition and without the addition of Agar.

Extraction steps

Ten grams of powder was added to 100ml of MRS broth media, and the same amount was added to another 100ml of Czapek Dox broth media. Then, all flasks were autoclaved and 1ml of *B. lactis* was inoculated to the autoclaved MRS broth and the same for *L. lactis.* Additionally, 1 ml of *C. utilis* was inoculated to the autoclaved czapek Dox broth and the same for *S. cerevisiae*. All samples were incubated for 7 days at 37ºC at 100 rpm.

3.6 Novel-mixed method using both natural and biological methods for getting the highest yield of astaxanthin

Crayfish powder (10g) was mixed with 20ml of flaxseed oil at the beginning of the experiment for 0 hour, another 10g was treated by the accumulative method. MRS and Czapek Dox broths were prepared in the same way, and then 2.5 ml of the astaxanthin containing oil of each time interval was inoculated to each broth (100 ml), using a bacterial filter for sterilization instead of autoclaving. Then, 1 ml of *B. lactis* was inoculated to "MRS broth + the 0 hours oil" and "MRS broth + the accumulative method oil". Afterwards, a volume of 1ml of *L. lactis* was inoculated to "MRS broth + the 0 hour oil" and "MRS broth + the accumulative sample oil". For the fungi strains, 1ml of *C.utilis* was inoculated to "Czapek dox broth + the 0 hours oil" and "Czapek dox broth + the accumulative method oil". Then, 1 ml of *S. cerevisiae* was inoculated to "Czapek dox broth + the 0 hours oil" and "Czapek dox broth + the accumulative sample oil".
All samples were incubated for 7 days at 37ºC at 100 rpm.

Chemical method for astaxanthin extraction

Using hexane and isopropanol in the ratio of (1:1) was determined following the method of **Sachindra *et al*. (2006)**. All samples of the biological method and the novel method were subjected to cooling lyophilization; the chemical method sample was evaporated using a rotary evaporator.

Spectrophotometer assay for astaxanthin (Sachindra *et al*., 2006)

The powder of each sample was diluted using petroleum ether (samples of the natural method were diluted with petroleum ether directly without lyophilization or evaporation); then the absorbance was measured at a wavelength of 468nm using the spectrophotometer with the following equation, and the carotenoids' yield was calculated only as astaxanthin.

$$\frac{V \times A \times dilution\ factor}{w \times 0.2}$$

Where; "V" is the extract volume, "A" is the absorbance at 468nm, "0.2" is the absorbance of 1 µg/L of the astaxanthin standard, and "W" is the sample's mass in grams.

Statistical analysis

Data presented in each experiment were means of triplicate assays. The SPSS 25 software was used in the determination of standard error (SE) ($p < 0.001$).

RESULTS

The concentration of astaxanthin extracted by flaxseed oil alone was gradually increased as the time interval increased. The highest concentration was observed in the accumulated method after 48 hours (27.5 µg/g), while its lowest was recorded in the zero hours (17 µg/g) as shown in Fig. (1). On the other hand, in the biological method of astaxanthin extraction by the fungal probiotics, the concentration of astaxanthin extracted by *S. cerevisiae* was higher (467.5 µg/g) than that extracted by *C. utilis* (290 µg/g); while, the extraction of astaxanthin by bacterial probiotics was (395 µg/g) in the case of *L. lactis*, which was higher than that extracted by *B. lactis* (320 µg/g) as shown in Fig. (2).

To achieve the higher concentration of astaxanthin, a mixed novel method was performed at both time intervals (zero hour and the accumulated method after 48 hours). The concentrations of astaxanthin at zero time were 2620, 1920, 1220, and 660 µg/g, extracted by *L. lactis*, *B. lactis*, *S. cerevisiae*, and *C. utilis*, respectively, as shown in Fig. (3).

Fig. (4) shows that, the concentrations of astaxanthin at the accumulated method after 48 hours were 19020, 17400, 12480, and 4620 µg/g, extracted by *L. lactis*, *B. lactis*, *S. cerevisiae*, and *C.utilis*, respectively, in comparison with the chemical method which yielded (320 µg/g).

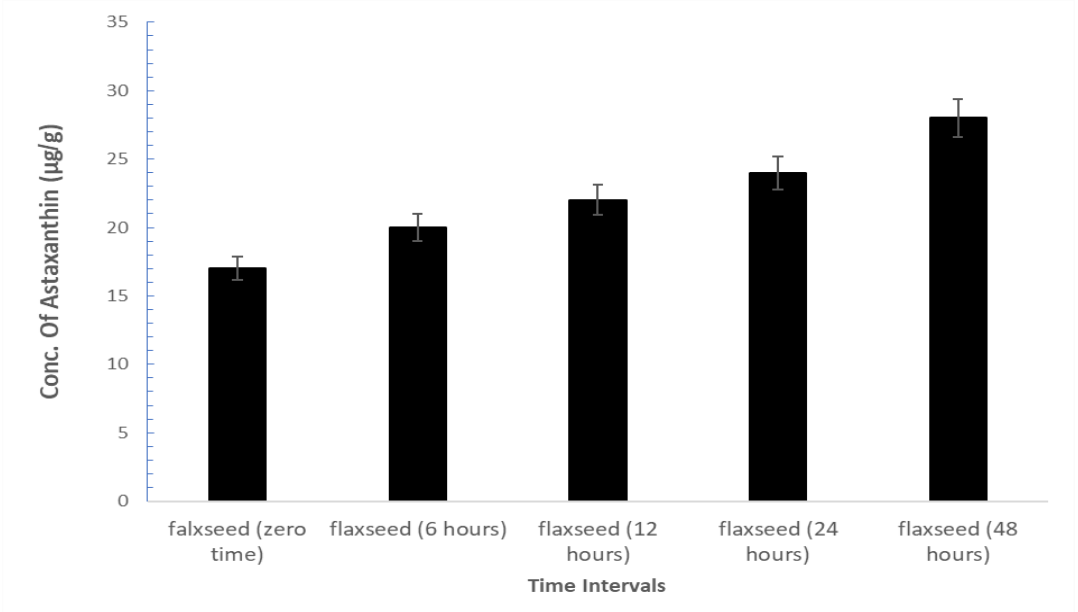

Fig. 1. The concentration of astaxanthin extracted by flaxseed oil alone at different time intervals (zero, 6, 12 and 24 hours) and accumulated method after 48 hours

Error Bars showed Mean ± SE ($p < 0.001$).

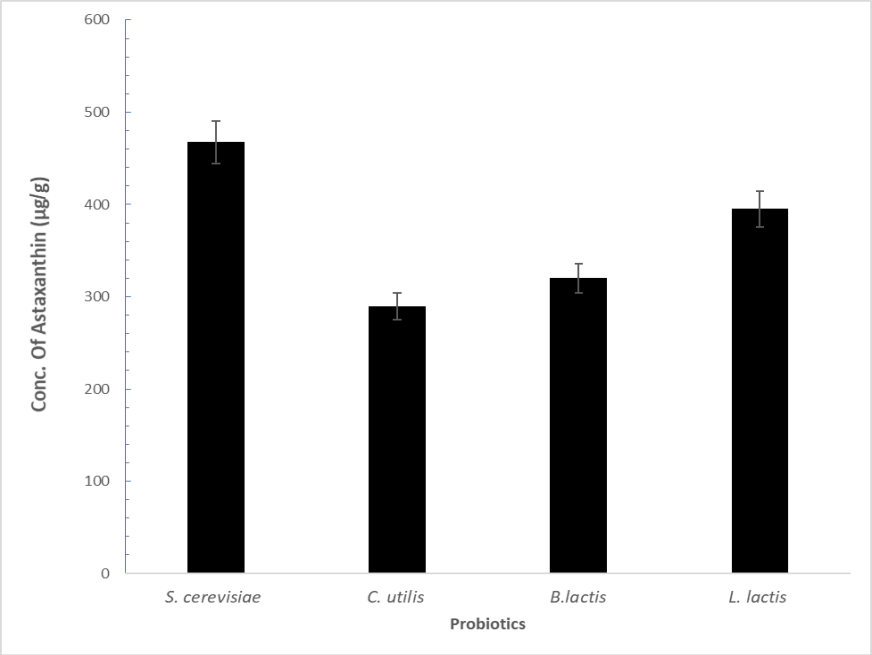

Fig. 2. The concentration of astaxanthin extracted by fungal probiotics (*S. cerevisiae – C. utilis*) and bacterial probiotics (*B. lactis – L. lactis*)

Error Bars show Mean ± SE ($p < 0.001$).

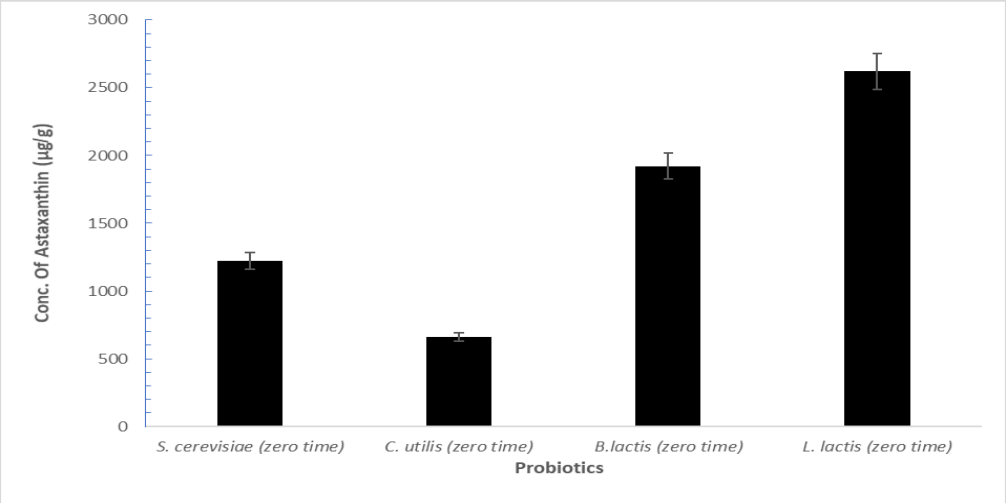

Fig. 3. The concentration of astaxanthin extracted by flaxseed oil and fungal probiotics (*S. cerevisiae – C. utilis*) with bacterial probiotics (*B. lactis – L. lactis*) at zero time

Error Bars show Mean ± SE ($p < 0.001$).

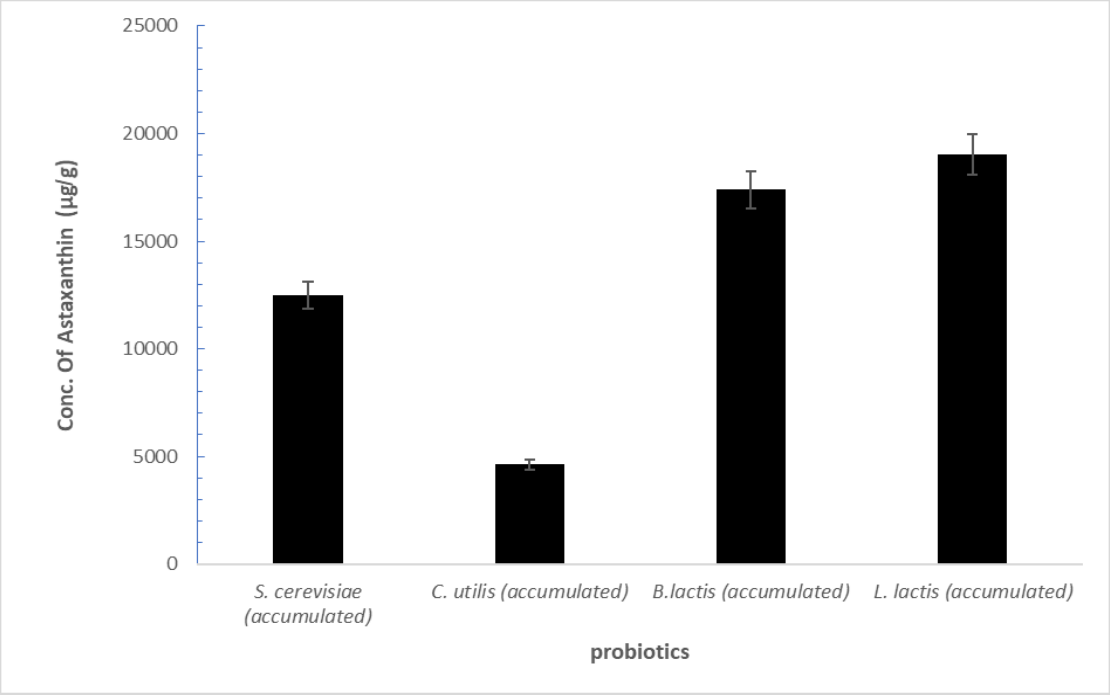

Fig. 4. The concentration of astaxanthin extracted by flaxseed oil and fungal probiotics (*S. cerevisiae – C. utilis*) with bacterial probiotics (*B. lactis – L. lactis*) at accumulated method after 48 hours. Error Bars show Mean ± SE ($p < 0.001$).

DISCUSSION

The consumption of astaxanthin as an animal food colorant and fish feed was approved by the United States Food and Drug **(Kishimoto *et al*., 2016)**. The usage of natural astaxanthin as a dye in the food industry was approved by European Commission. Recent findings on the potential effects of astaxanthin and its esters on biological activities were reported. The use of astaxanthin as a nutritional supplement has been rapidly growing in foods, feeds, nutraceuticals and pharmaceuticals. Astaxanthin has many biological activities and health benefits. It may be used for the prevention of various diseases and utilized in commercial applications as well. Astaxanthin production from natural sources is among the most successful biotechnological activities. Astaxanthin has great demand in food, feed, nutraceutical and pharmaceutical applications **(Yamashita, 2005)**. This significance has promoted major efforts to improve astaxanthin production from biological sources instead of synthetic ones including crawfish exoskeleton by-product sources. On the other hand, since the extraction occurs by biological eco-friendly method, the product will be hundred percent natural.

Astaxanthin has many medical importance such as human food supplement. Its importance is attributed to its anticancer **(Jyonouchi *et al*., 2000)**, antioxidant **(Naguib, 2000)**, anti- inflammatory **(Ohgami *et al*., 2003)**, antiaging **(Tominaga *et al*., 2012)** and

anti-infection activities. In the present study, different methods for astaxanthin extraction were used. First method was the natural one, in which astaxanthin was extracted using flaxseed oil solely, with taking time intervals in consideration. However in preceeding methods, astaxanthin was extracted using flaxseed oil and ethanol combined **(Scott, 2020)**. Astaxanthin concentration obtained by the present natural accumulated method after 48 hours was 27.5 µg astaxanthin/g sample, which is approximately similar to the result of **Pu and Sathivel (2011)** (3.02 g astaxanthin/100 g sample) using flaxseed oil and crayfish waste. In the second extraction method, the biological method bacterial (*L. lactis* and *B. lactis*) and fungal (*S. cerevisiae* and *C. utilis*), probiotics were used for astaxanthin extraction. In the present study, fungal probiotics were used for the astaxanthin extraction for the first time giving a promising result. While in the preceeding biological extraction methods, only bacterial strains were used for the astaxanthin extraction **(Armenta-López *et al*., 2002; Khanafari *et al*., 2007; Prameela *et al*., 2017)**. In the present biological method, the concentration of astaxanthin extracted by bacterial probiotics showed higher concentration using *L. lactis* (395 g astaxanthin/100 g sample), compared to that when *B. lactis* (320 g astaxanthin/100 g sample) was used. Whereas, the concentration of astaxanthin extracted by fungal probiotics recorded higher concentration using *S. cerevisiae* (467.5 g astaxanthin/100 g sample) than using *C. utilis* (290 g astaxanthin/100 g sample). In general, the current recorded astaxanthin concentration extracted by this method is significantly higher than the method of astaxanthin bacterial extraction **(Armenta-López *et al*., 2002)** for shrimp waste, where the concentration was 1.25 µg astaxanthin/g sample. The third method, which is the mixed-novel method was used for the first time in the present study. It showed the highest concentration of astaxanthin compared to the other methods. This may be due to using flaxseed oil combined with bacterial fungal probiotics for astaxanthin extraction. Flaxseed oil in the accumulated method after 48 hours, aligned with bacterial and fungal probiotics (*L. lactis, B. lactis, S. cerevisiae, and C. utilis*) yielded high concentration of astaxanthin (19020 µg/g, 17400 µg/g, 12480 µg/g and 4620 µg/g, respectively). The final extraction method is the chemical method. In the present study, the astaxanthin concentration extracted by chemical method was 320 µg astaxanthin/g sample, which is significantly higher than the highest concentration of carotenoids obtained from the chemical method approached in the study of **Sachindra *et al*. (2005)** on shrimp waste (43.9 µg astaxanthin/g sample). Furthermore, the present result is higher than the highest astaxanthin concentration (72.0 µg astaxanthin/g sample) recorded in the work of **Li *et al*. (2017)** who extracted astaxanthin from shrimp waste using ethanol and high pressure.

CONCLUSION

The biological and novel mixed-method are more efficient in astaxanthin extraction, and the best method of high yield astaxanthin extraction is the mixed-novel method at 48

hours accumulated method, where astaxanthin-containing oil was treated with *Lactobacillus lactis* (present in yogurt)

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