Optimization of Enzymatic Extraction of Astaxanthin from *Xanthophyllomyces dendrorhous* Using a Recombinant Cellulase

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ABSTRACT

The Study on the Utilization of Valuable Components Derived from Various Biomass Byproducts Through Saccharification and Fermentation Extracts and Microbial Biomass

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In the food industry, interest in and consumption of health-functional foods for antioxidants and immune enhancement are increasing significantly due to aging and the emergence of novel infectious diseases. As a result, research on functional compounds is being actively conducted. Astaxanthin, a ketocarotenoid-type red pigment, has been studied for its potent antioxidant properties, which are approximately ten times stronger than those of other carotenoids. However, the chemical form of astaxanthin has lower bioavailability and stability compared to natural extracts. In this study, a cellulase enzyme for degrading the cell wall of *Xanthophyllomyces*

dendrorhous was developed using a recombinant microorganism. Additionally, astaxanthin extracted from *X. dendrorhous* was analyzed using HPLC. As a result, enzyme production was successfully achieved in the recombinant microorganism, and the enzymatic activity of the prepared enzyme was confirmed both qualitatively and quantitatively. The enzyme reaction method, which yielded an astaxanthin concentration of 0.04966 mg/mL, was identified as a more suitable extraction approach compared to the soy oil mixing method, which resulted in a lower concentration of 0.00251 mg/mL. Through these findings, the extraction method using enzymatic reactions is expected to be directly applicable to the functional food market for products containing astaxanthin extracted from *X. dendrorhous*.

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Ⅰ. INTRODUCTION

Astaxanthin is one of the carotenoid pigments, primarily responsible for the characteristic red color found in salmonids and crustaceans. Carotenoids comprise a series of over 600 pigments and can be synthesized in various organisms, including mosses, bacteria, fungi, algae, and plants (Goodwin, 1980). The structure of carotenoids is derived from lycopene and consists of a hydrocarbon form with 40 carbon atoms. This structure is connected through a conjugated double-bond system or a polyene system formed by the fusion of two terminal units. Astaxanthin contains 11 double bonds, which are conjugated, and has two cyclohexene rings at both ends of the molecule, providing stability Higuera-Ciapara et al., 2006). Due to its outstanding antioxidant properties, astaxanthin has significant value in the health supplement market. While synthetic astaxanthin is widely used in the global market, interest in natural pigments has been increasing in recent years. Natural astaxanthin can be extracted from sources such as the green microalga *Haematococcus pluvialis*, the red yeast *Phaffia rhodozyma*, and crustacean by-products (Higuera-Ciapara et al., 2006). Synthetic astaxanthin has the same molecular structure as naturally occurring astaxanthin and is one of the primary carotenoids used in the global aquaculture industry. However, due to the growing demand for natural compounds and the high cost of synthetic astaxanthin, the industrialization of natural astaxanthin production has gained more attention (Lu et al., 2021). The biosynthetic pathway of astaxanthin has been extensively studied in algae, bacteria, yeast, and other eukaryotic organisms. Among microbial sources, species that can economically

compete with synthetic astaxanthin include the green microalga *H. pluvialis*, the red yeast *P. rhodozyma*, and *Xanthophyllomyces dendrorhous*. However, due to limited production capabilities, these natural products currently occupy only a small portion of the market (Zheng et al., 2006).

Currently, various research reports have been conducted on *H. pluvialis*, a microalga that produces astaxanthin (Kakizono et al., 1992). In addition to *H. pluvialis*, the yeast *X. dendrorhous* is also a microbial source that naturally produces astaxanthin. *H. pluvialis* has a thick cell wall containing mannan, requiring a pre-treatment process to break the cell wall (Hagen et al., 2002). This is typically done using methods such as organic solvent extraction, ultrasonic extraction, and supercritical carbon dioxide extraction. Since astaxanthin production in *H. pluvialis* is influenced by environmental stress, optimizing culture and extraction conditions is essential. On the other hand, *X. dendrorhous*, being a yeast, has a cell wall composed of chitin and beta-glucan. Although it is relatively more flexible than microalgae, pre-treatment is still required for extraction. Astaxanthin extraction from yeast is carried out through methods such as hot water extraction and organic solvent extraction (Artigas-Hernández et al., 2023). Since yeast cells are relatively easy to break, physical methods can also be used. Additionally, enzymatic methods using cellulase or protease can be applied to degrade the cell wall and facilitate astaxanthin release (Dong et al., 2014). Compared to microalgae, yeast has simpler culture conditions and a faster growth rate, making it suitable for large-scale production and economically viable (Basiony et al., 2022). The astaxanthin extraction process varies depending on the

physiological characteristics and cell structure of each strain, leading to differences in utilization efficiency in the microbial industry. Increasing astaxanthin concentration through microbial fermentation is a crucial factor in competing with synthetic production, which currently serves as the primary source in the market (Rodríguez-Sáiz et al., 2010).

However, physical extraction methods such as ultrasonic extraction also have limitations that need to be addressed. Due to the heat sensitivity of functional compounds like astaxanthin, the heat generated during the ultrasonic extraction process can lead to the degradation of heat-sensitive molecules (Mehta et al., 2022). Additionally, certain solvents may hinder the effective extraction of astaxanthin, and the toxicity of the solvents must also be considered (Bello et al., 2025). According to reference studies, a mixture of ethanol and ethyl acetate has been used as an extraction solvent (Rajendran et al., 2023) (Zou et al., 2013). While ethanol and ethyl acetate may pose environmental and toxicity issues, alternative approaches are being explored, including environmentally conscious techniques like ultrasound-assisted extraction and enzyme (Rajendran et al., 2023). To address these challenges, research on environmentally friendly and non-toxic alternative solvents is necessary, or optimization of solvent mixing ratios should be pursued (Chemat et al., 2019) (Abolore et al., 2024). Although ultrasound-assisted extraction is a highly useful method for astaxanthin extraction, various approaches are needed to compensate for its drawbacks, such as heat sensitivity, solvent selection, and efficiency variability. Therefore, applying biological methods is expected to further enhance the extraction efficiency of astaxanthin. Interest in

health-functional foods, particularly for antioxidants and immune enhancement, is rising due to aging and new infectious diseases. Astaxanthin, a potent antioxidant found in *X. dendrorhous*, is being researched for its benefits, although its chemical form has lower bioavailability. This study developed a cellulase enzyme using a recombinant microorganism to degrade the cell wall of *X. dendrorhous* and analyzed the extracted astaxanthin using HPLC. The enzyme reaction method produced a higher concentration of astaxanthin (99%) compared to the soy oil mixing method (5%), suggesting its potential for the functional food market.

Ⅱ. MATERIALS AND METHODS

2.1. Strains, Plasmids, and Media

For the purposes of recombinant DNA manipulation and gene expression, the *Escherichia coli* strains DH5α and BL21(DE3) were utilized, as specified in Table 1. *E. coli* was cultured at 37 °C in Luria-Bertani (LB) medium, which consists of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride, and was supplemented with 50 μg/mL ampicillin. *X. dendrorhous* (strain information: KCCM 50183, ATCC 24202) was grown in YM broth (BD/Difco, Yeast Mold Agar/Broth was prepared according to the composition published by Wickerham, who suggested using YM broth acidified to a pH of 3.0-4.0 as a growth medium for yeast from communities containing both yeasts and molds.).

Table 1. Microbial strains and plasmids used in this study

Strain or plasmid	Genotype or construct	Reference or source [a]
Bacterial strains		
Escherichia coli DH5α	F^-, *deoR*, *endA1*, *gyrA96*, *hsdR17*(rk^-mk^+), *recA1*, *relA1*,*supE44*, *thi-1*, Δ(*lacZYA-argF*)*U169*, (Phi80*lacZ*delM15)	Invitrogen (Carlsbad, CA, USA)
Escherichia coli BL21 (DE3)	F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*]	Invitrogen (Carlsbad, CA, USA)
Clostridium cellulovorans	WT strain ATCC 35296	ATCC [a]
Xanthophyllomyces dendrorhous	WT strain ATCC 24202	ATCC [a]
Transformants		
E. coli BL21 (pET22b (+) Control)	[$T7_P$-*pelB*-$T7_T$]	This study
E. coli BL21 (pET22b (+) EngD)	[$T7_P$-*pelB*-*engD*-$T7_T$]	This study
Plasmids		
pET22b (+)	$T7_P$-*pelB*-$T7_T$	Novagen (San Diego, CA, USA)
pET22b (+) EngD	$T7_P$-*pelB*-*engD*-$T7_T$	This study

2.2. Culture and Growth Curve

In the study, the yeast strain *X. dendrorhous* was subjected to white light irradiation to establish more efficient growth conditions for astaxanthin extraction. For optimizing the culture conditions, experiments were conducted based on previous research (Harith et al., 2020) (Harith et al., 2020), utilizing white light. For the YM broth, 0.9 g of yeast and 1.5 g of peptone were added to 292.5 ml of sterilized water, then 7.5 ml of pre-sterilized glucose (20 g/100 ml) was added to prepare the medium based on 300 ml total volume. Similarly, for the YM agar, 4.5 g of agar was added to prepare the medium. The powdered *X. dendrorhous* strain was dissolved in saline and streaked onto YM agar. The culture was incubated for more than three weeks until the red color of astaxanthin became prominent. Colonies were then inoculated into 5 ml of YM broth. After 24 hours of shaking at 20 °C, a main culture was established, and samples were irradiated with white light for 4 to 9 days. The optical density was measured to confirm the growth of the strain.

2.3. Expression and Purification of Recombinant Proteins

To produce a cell wall-lysing enzyme, the EngD gene (approximately 111.8 kDa) from *Clostridium cellulovorans* was cloned into the pColdII vector and transformed into *E. coli* BL21. Colony PCR, using bacterial genomic DNA as a template, was performed with diluted colonies or liquid cultures (1:10 dilution in D.W. for liquid cultures, and dilution in physiological saline or D.W. for colonies) to avoid template overload; PCR products were analyzed by electrophoresis (1% agarose gel, 15 min) and purified using a PCR purification kit (COSMOgenetech PCR Clean-up kit). Faint bands were re-amplified with minimized electrophoresis time (0.8% agarose gel) to prevent DNA degradation. Purified PCR products and the pColdII vector were digested with restriction enzymes (SacI and KpnI) at 37°C for 2 hours. Following digestion, DNA fragments were separated by electrophoresis (0.8% agarose gel, 15 min), and the target DNA band was excised and purified using a gel extraction kit (COSMOgenetech PCR Clean-up kit). DNA concentrations were determined using a Nanodrop spectrophotometer (optimal concentration: 10-20 ng/μL). Ligation was performed using the EZ-Fusion™ HT Cloning Kit (enzynomics), with a vector:insert ratio of 1:2 and a target mass of 75-100 ng in a 10 μL reaction, incubated at 50°C for 15 minutes. Ten microliters of the ligation reaction were transformed into 150 μL of competent *E. coli* cells (Rosetta), with positive and negative controls. Cells were incubated on ice, heat-shocked at 42°C for 90 seconds, and then incubated in LB broth at 37°C with shaking, followed by plating on LB agar containing ampicillin and incubation at 37°C for 24 hours. Colony PCR was performed to

confirm successful transformation, and the resulting recombinant *E. coli* strain was used for enzyme production. To prepare for the inoculation and expression of proteins, *E. coli* strains were grown by shaking at 200 rpm in Luria–Bertani (LB) medium, supplemented with 50 μg/mL ampicillin. The cultures, housed in 15 mL conical tubes and 500 mL Erlenmeyer flasks, were incubated with shaking at 37 °C. Upon reaching an optical density at 600 nm of 0.6, the culture was cooled to 16 °C. Subsequently, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium for overnight induction under the control of the T7 promoter.

Post-induction, cells were collected by centrifugation at 4,000 x *g* for 10 min and washed twice. The cell pellet was resuspended in 10 mL of ice-cold lysis buffer (50 mmol L^{-1} NaH_2PO_4, 300 mmol L^{-1} NaCl, and 10 mmol L^{-1} imidazole, pH 8.0). Cell disruption was performed using sonication, and the resulting lysate was centrifuged at 10,000 x *g* for 30 min. The supernatants containing crude cell extracts were then applied to a Ni-NTA column for binding of His-tagged recombinant proteins. The column was cleansed with a wash buffer (50 mmol L^{-1} NaH_2PO_4, 300 mmol L^{-1} NaCl, and 20 mmol L^{-1} imidazole, pH 8.0). The bound recombinant proteins were eluted using an elution buffer (50 mmol L^{-1} NaH_2PO_4, 300 mmol L^{-1} NaCl, and 250 mmol L^{-1} imidazole, pH 8.0). The eluted proteins were then dialyzed against a 20 mmol L^{-1} Tris-HCl buffer (pH 8.5). Furthermore, the culture supernatant was concentrated using ultrafiltration through centrifugal filter units with a 10 kDa cutoff membrane. The protein concentration was determined using the Bradford method, employing a Quick Start protein assay kit and using bovine serum

albumin as the standard. The prepared enzyme protein samples were separated by size using SDS-PAGE gel electrophoresis, running for approximately 30-45 minutes, and following electrophoresis, Western blotting was performed, utilizing the InVision His-tag In-gel Stain during the Western blot procedure; after the Western blot, the membrane was visualized using a Chemidoc imaging system, and the gel was subsequently stained with Coomassie Brilliant Blue for approximately 15 minutes, and the resulting protein bands were then compared to a molecular weight standard to verify the size and position of the target protein. This experiment was conducted in triplicate.

2.4. Astaxanthin Extraction

For astaxanthin extraction, the *X. dendrous* strain was inoculated in YM broth and centrifuged to remove the supernatant, obtaining the pellet. Based on a 1000 μL pellet solution, the highest concentration of astaxanthin was extracted when a sodium citrate aqueous solution and cellulase enzyme solution were used at a ratio of 1:4, with a 6-hour enzymatic reaction and two consecutive extractions. For safety considerations during the extraction process, 99% acetone was replaced with 99% ethanol, which yielded similar results, leading to the use of 99% ethanol. Additionally, when NaCl solution was removed to facilitate drying, the results remained consistent, so the extraction was conducted without adding NaCl solution. When these variables were applied to design an efficient extraction process, the enzymatic reaction method was determined to be a suitable extraction approach. The detailed extraction conditions were as follows: Based on a 1000 μL pellet solution, 3 mL of sodium citrate aqueous solution and 1 mL of enzyme solution were added, followed by enzymatic reaction at 37°C, 200 rpm for 6 hours with stirring. The pellet was then washed, and 1 mL of 99% ethanol was added, followed by stirring under the same conditions for 10 minutes, after which the supernatant was collected by centrifugation. An additional 1 mL of 99% ethanol was added, and the process was repeated under the same conditions, with the supernatant collected again. The collected samples were transferred to a plate, dried under a clean bench using 99% ethanol, and dissolved in 100 μL of soy oil for further use. To optimize astaxanthin extraction, a soy oil stirring method was implemented, referencing the

protocol described by Shengzhao Dong et al. (Dong et al., 2014). A ratio of wet microbial pellet to soy oil of 2 g : 16 mL was used. Soy oil was added to the microbial pellet, which had been exposed to white light, at the specified ratio, followed by vortexing. The mixture was then transferred to a 100 mL beaker and stirred using a magnetic stirrer at 500 rpm at room temperature (RT) for at least 2 hours. To enhance cell disruption, the mixture underwent two cycles of sonication, each consisting of a 60-second pulse at 90% power followed by a 30-second incubation on ice. This cycle was repeated eight times. The resulting extract was then purified using a syringe filter, and the absorbance was measured. Based on these parameters, an efficient extraction method was designed. To determine the most suitable extraction conditions, the soy oil stirring method and the enzymatic reaction method were compared under identical mild conditions, and the astaxanthin yield from each method was quantified to optimize the extraction process.

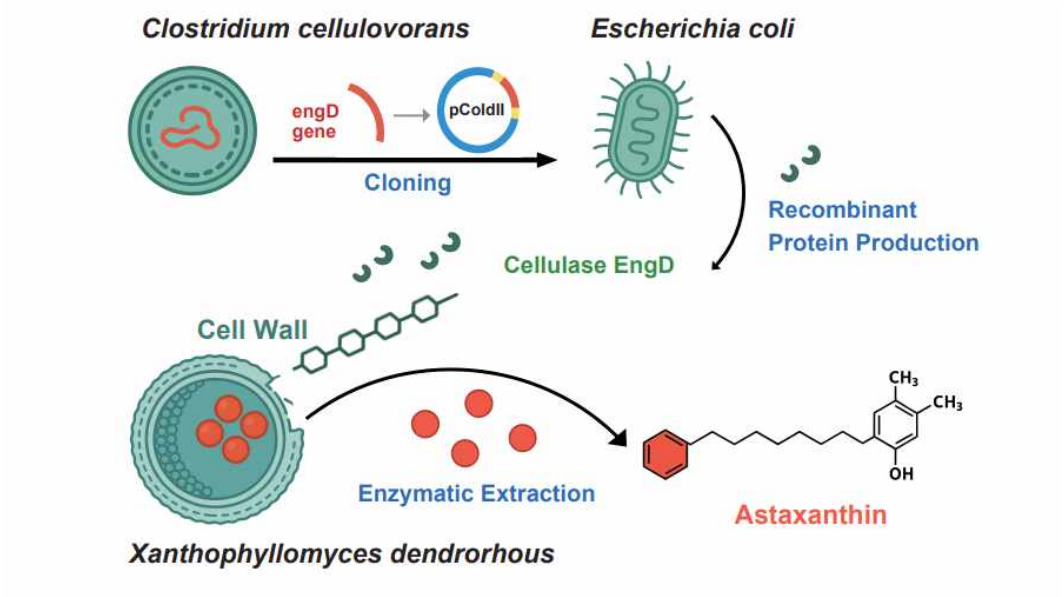

Fig. 1. Schematic diagram of the experiment.

2.5. High Performance Anion-exchange Chromatography

This experiment aimed to quantify the astaxanthin content in samples obtained from the extraction of astaxanthin from the *X. dendrorhous* strain using HPLC analysis. Standard reagents were prepared using SuperPure Astaxanthin from The Synergy Company. This product is available in capsule form, containing a powder extracted from 120 mg of *H. pluvialis algae*. It specifies that the mass of the powder corresponding to one serving is 0.56 g, with an astaxanthin content of 6 mg per serving. The powder was dissolved in 10 mL of vegetable oil, such as canola oil, and then diluted to concentrations of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. After filtering through a syringe filter to minimize bubbles, the solution was transferred to vials for use as standard reagents. For the HPLC mobile phase, a methanol-water mixture (97:3, v/v) was used. This solution was filtered through a membrane, degassed using an ultrasonic device, and then employed for analysis. The HPLC conditions included the use of a C18 column and a DAD (UV) detector. The analysis was conducted at room temperature with a flow rate of 1.0 mL/min, measuring at a wavelength of 474 nm. The measurement samples were prepared by applying the enzymatic reaction method to the culture medium of *X. dendrorhous*, based on the work of previous research (Bey et al., 2016).

Ⅲ. RESULTS AND DISCUSSION

3.1. Strain Cultivation and Astaxanthin Production Under Light Exposure

To establish optimal culture conditions, *X. dendrorhous* colonies were cultured in YM broth for over three weeks until a distinct red color of astaxanthin became evident. The experimental group was then exposed to white light and cultured under the same conditions for 4 to 9 days, during which the optical density was measured (Fig. 2). The graph presents experimental results comparing the optical density (OD) of the culture medium when exposed to white light versus the control condition, to optimize astaxanthin production from *X. dendrorhous*. The optical density at 600 nm (OD600) of the culture medium, which was cultivated in 300 mL of YM broth under white light exposure at 20°C and 200 rpm shaking incubation, was measured every 24 hours, and the results are shown in the graph. By comparing the OD values up to the fourth day, it was observed that the OD under white light exposure was higher than that of the control group. This difference suggests that astaxanthin production within *X. dendrorhous* was sufficiently achieved over the four-day period in 100 mL of YM broth. However, when the experiment was continued until the ninth day, the OD values under white light exposure were lower than those of the control.

Despite this, when astaxanthin extraction experiments were conducted afterward, a higher concentration of astaxanthin was extracted

from the samples exposed to white light. Since the OD values up to the fourth day were higher and the astaxanthin extraction experiment also yielded a significantly higher concentration compared to the control, it can be concluded that white light exposure had a positive effect on optimizing culture conditions. For practical application in industrial settings, future experiments involving large-scale cultivation should be conducted. Accordingly, it will be necessary to adjust factors such as the optimal cultivation period and the intensity of white light depending on the scale-up process (Harith et al., 2020). The research on these cultivation conditions is expected to enhance both the productivity and economic viability of astaxanthin, thereby improving its market potential.

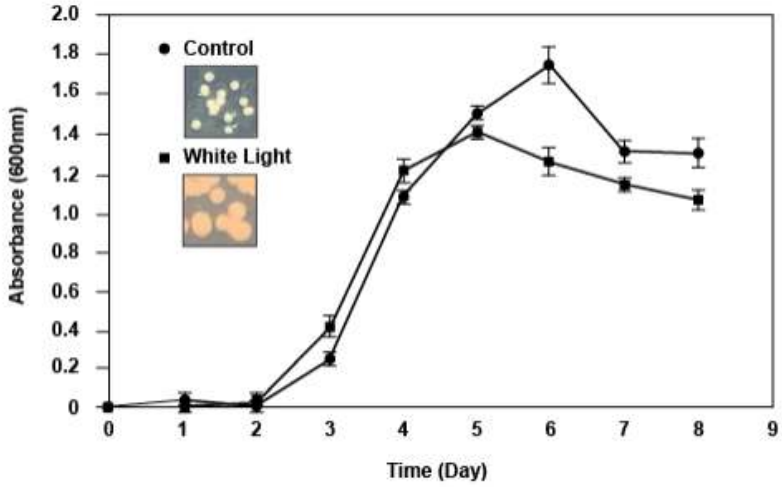

Fig. 2. Growth and pigment production in *X. dendrorhous*. Growth was measured by the absorbance at 600 nm, which is represented by solid line, at different culture conditions such as (closed circle) control and (closed square) white light. Growth phenotype of *X. dendrorhous* strains at different culture conditions indicate pigment production level by color intensity.

3.2. Enzyme Production Using Recombinant Microorganisms

Cellulase EngD is derived from *C. cellulovorans*. noncellulosomal cellulases like EngD lack dockerin domains but possess cellulose-binding domains (CBDs) (Murashima et al., 2003). Furthermore, a study evaluating the enzymes and reaction conditions affecting astaxanthin release indicates that enzymatic degradation of cell wall polysaccharides can facilitate astaxanthin release (Zhao et al., 2019). Therefore, Cellulase EngD, with its CBD, exhibits affinity for cellulose, and this enzyme may aid in astaxanthin release through enzymatic degradation of cell wall polysaccharides. To produce an enzymatic protein capable of effectively lysing the cell wall of the target microorganism, *E. coli* BL21 (pET22b (+) EngD) strain was used as a recombinant microbial strain, and the results were analyzed. After culturing the strain and performing a SDS-PAGE, the results are shown in Figure 3. The molecular weight of EngD is 55.9 kDa, and a clear band corresponding to the target protein was observed in EngD. This indicates that the target protein was expressed in a significant amount. The band corresponding to the target protein appeared within the expected size range, and the noise-like protein bands appeared relatively faint. This suggests that the gene's protein expression was sufficiently achieved. Based on these results, if the strain producing the target protein is cultivated on a large scale, followed by extraction and protein concentration, it is expected that the enzyme could effectively lyse microbial cell walls and be applied to astaxanthin extraction.

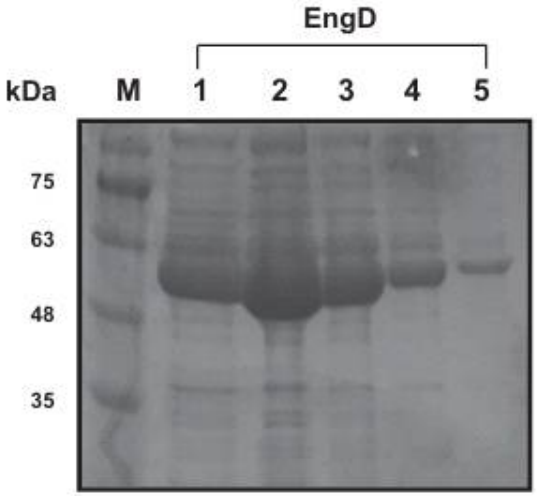

Fig. 3. Confirmation of protein expression through SDS-PAGE analysis for EngD. Lanes: M, protein marker; 1-5, purified cellulase EngD.

3.3. Comparison of the Soy Oil Method and Enzyme Method

After extracting astaxanthin from the *X. dendrorhous* strain, an HPLC analysis was conducted to verify whether the composition of the sample was like that of a commercially available astaxanthin standard reagent. The experiment involved measuring samples using HPLC, including distilled water as a buffer, standard reagent solutions at various concentrations, and samples extracted from *X. dendrorhous*. The results confirmed that no peaks were observed in the distilled water, indicating that it was an appropriate buffer. The HPLC analysis of the standard reagent and extracted samples showed a distinct peak at about 6 minutes, indicating the presence of astaxanthin (Fig. 4).

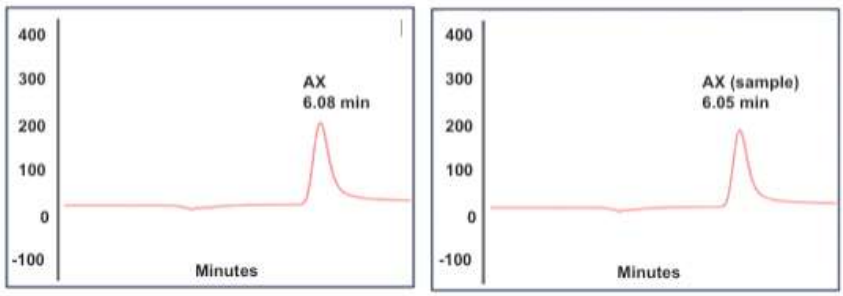

Fig. 4. HPLC analysis of astaxanthins produced in yeast. In the peak data below, the left line is the peak of the astaxanthin standard, and the right line is the peak of astaxanthin produced in the strain.

Additionally, when experimental standard astaxanthin and extracted samples were analyzed using the mobile phase, the peak of the standard reagent also appeared at approximately 6 minutes. This provided numerical confirmation that the sample extracted from *X. dendrorhous* contained astaxanthin.

For the comparative evaluation of enzymatic activity between the soy oil mixing method and the enzyme reaction method, cell disruption was performed twice at a wet pellet-to-soy oil ratio of 2 g:16 mL. The disruption process involved sonication at 90% power for 60 seconds, followed by 30 seconds on ice, repeated eight times. The sample was then purified using a syringe filter, and absorbance was measured (Fig.5).

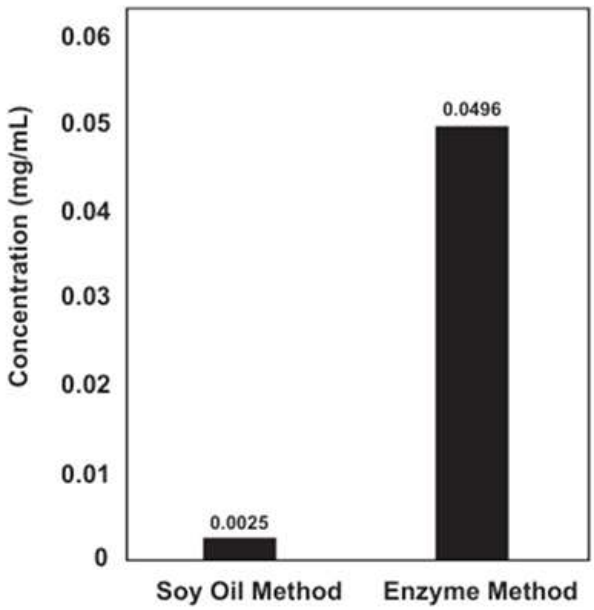

Fig. 5. Extraction of astaxanthins from yeast biomass using different methods. Comparison between soy oil method and enzyme method. Data represent the mean of triplicate reactions, with error bars indicating standard deviations.

The astaxanthin concentration was determined to be 0.00251 mg/mL, indicating a significantly lower extraction yield compared to the enzyme reaction method. Even when increasing the number of sonication cycles and duration, astaxanthin remained in the pellet and did not mix well with soy oil, leading to phase separation. The same result was observed when replacing the organic solvent used for cell disruption from 99% ethanol to 99% acetone. Additionally, prolonging the mixing time with soy oil did not extract the remaining astaxanthin, and most of the sample was lost due to the syringe filter. Despite modifying these variables to design an efficient extraction process, the soy oil extraction method was deemed unsuitable. Given that astaxanthin is positioned at the hydrophilic ends of the phospholipid bilayer, linking the bilayer structure of the cell membrane, the soy oil extraction method was not an appropriate approach.

For the enzyme reaction method, the highest astaxanthin concentration was extracted when a 1000 μL pellet solution was treated with a sodium citrate aqueous solution and cellulase enzyme solution at a ratio of 1:4, followed by a 6-hour enzymatic reaction and two consecutive extractions. After extracting astaxanthin from a 4 mL pellet sample, drying it with 99% ethanol, and dissolving it in 100 μL of soy oil, the concentration was measured (Fig. 5). The astaxanthin concentration was determined to be 0.04966 mg/mL, indicating a significantly higher extraction yield compared to the soy oil mixing method.

For safety considerations, 99% acetone was replaced with 99%

ethanol during the extraction process, yielding similar results, leading to the use of 99% ethanol. Additionally, when NaCl solution was removed to facilitate drying, the results remained consistent, so the extraction was conducted without adding NaCl. When the enzyme reaction temperature increased to 45°C and the experiment was conducted under the same conditions, no significant difference was observed. Based on these findings, the enzyme reaction method was determined to be the most suitable extraction approach for efficient astaxanthin recovery. In the case of the Soy Oil mixing method, the astaxanthin concentration was measured at 0.00251 mg/mL through absorbance measurements and standard curve calculations, corresponding to 5%. In contrast, the enzyme reaction method yielded a concentration of 0.04966 mg/mL, confirming an extraction yield of 99%. This indicates that, under similar protocol conditions, the enzyme reaction method demonstrated a significantly higher extraction yield compared to the Soy Oil mixing method.

Ⅳ. CONCLUSION

The interest in functionality within the food industry is rapidly increasing. In the functional food market, there is also a growing movement to compensate for nutrient deficiencies by utilizing ingredients derived from natural sources (Granato et al., 2020). This study aimed to optimize the culture conditions of *X. dendrorhous* for the efficient extraction of natural astaxanthin and to develop an effective extraction method. The optimal culture conditions proposed involve exposing the culture to white light and conducting shaking incubation for a minimum of four days and up to nine days. For efficient astaxanthin extraction, the enzyme reaction method, which yielded a concentration of 99%, was identified as a more suitable approach compared to the soy oil mixing method, which resulted in a lower concentration of 5%

Through this microbial-based functional study, it is expected that the development of functional ingredients with broader applications will be possible. The objective of this study was to expand the use of astaxanthin in the functional food industry by designing the optimal culture conditions for *X. dendrorhous* and establishing an efficient extraction strategy for astaxanthin within *X. dendrorhous*. Additionally, as a related study, research was conducted to produce enzymes through microbial genetic engineering to achieve a more cost-effective enzymatic extraction method. The extracted samples were analyzed using HPLC to confirm the astaxanthin peak. As a result, in the enzyme protein production experiment, cellulase EngD demonstrated sufficient expression of the target protein.

Furthermore, HPLC analysis of the extracted samples confirmed the presence of astaxanthin. Additional studies were conducted to enhance enzyme activity and improve measurement accuracy by modifying the HPLC analysis conditions. The significance of this study lies in expanding research on optimal culture and extraction conditions, as well as developing other enzyme proteins for astaxanthin extraction from *X. dendrorhous*.

This study demonstrates the potential for commercializing enzyme proteins produced from recombinant microorganisms and directly applying astaxanthin extracted from *X. dendrorhous* to the functional food market. Building on this research, the feasibility of industrially applying enzymatic methods for the extraction of astaxanthin from *X. dendrorhous* can be evaluated. Enzymes exhibit high specificity for particular substrates, enabling the effective extraction of specific compounds such as astaxanthin (Harith et al., 2020). Furthermore, these processes typically occur at lower temperatures and pH levels, resulting in reduced energy consumption and minimizing the loss of heat-sensitive compounds, thereby enhancing the bioavailability and stability of astaxanthin. These advantages facilitate the establishment of relatively straightforward industrial setups. Recent studies have also proposed the use of recombinant microorganisms for the enzymatic extraction of astaxanthin, demonstrating that such enzymatic extraction methods are suitable for large-scale production and facility implementation. The enzymatic approach offers high productivity and minimal raw material loss, providing long-term economic benefits compared to initial investment costs. Additionally, the potential for enzyme reuse and process optimization can further reduce production costs (Storebakken et

al., 2004). By proposing the development of additional enzyme proteins for astaxanthin extraction from *X. dendrorhous*, this research suggests a more cost-effective and efficient production method, ultimately contributing to the industrial growth of the astaxanthin market.

REFERENCES

Goodwin, T. W. (1980). Nature and distribution of carotenoids. Food Chemistry, 5, 3-13. https://doi.org/10.1016/0308-8146(80)90061-8

Higuera-Ciapara, I., Félix-Valenzuela, L., & Goycoolea, F. M. (2006). Astaxanthin: a review of its chemistry and applications. Crit Rev Food Sci Nutr, 46, 185-96.

Lu, Q., Li, H., Zou, Y., Liu, H., & Yang, L. (2021). Astaxanthin as a microalgal metabolite for aquaculture: A review on the synthetic mechanisms, production techniques, and practical application. Algal Research, 54, 102178.

Zheng, Y. G., Hu, Z. C., Wang, Z., & Shen, Y. C. (2006). Large-Scale Production of Astaxanthin by Xanthophyllomyces Dendrorhous. Food and Bioproducts Processing, 84, 164-166.

Kakizono, T., Kobayashi, M., & Nagai, S. (1992). Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in a green alga, Haematococcus pluvialis. Journal of Fermentation and Bioengineering, 74, 403-405. https://doi.org/10.1016/0922-338x(92)90041-r

Hagen, C., Siegmund, S., & Braune, W. (2002). Ultrastructural and chemical changes in the cell wall of Haematococcus pluvialis (Volvocales, Chlorophyta) during aplanospore formation. European Journal of Phycology, 37, 217-226. https://doi.org/10.1017/S0967026202003669

Artigas-Hernández, D., Berzosa, A., Aguilar-Machado, D., Raso, J., & Artal, M. (2023). Using eutectic solvents for extracting astaxanthin from dry biomass of Xanthophyllomyces dendrorhous pretreated by pulsed

electric fields. Separation and Purification Technology, 324, 124496. https://doi.org/10.1016/j.seppur.2023.124496

Dong, S., Huang, Y., Zhang, R., Wang, S., & Liu, Y. (2014). Four Different Methods Comparison for Extraction of Astaxanthin from Green Alga Haematococcus pluvialis. The Scientific World Journal, 2014, 694305. https://doi.org/10.1155/2014/694305

Basiony, M., Ouyang, L., Wang, D., Yu, J., Zhou, L., Zhu, M., Wang, X., Feng, J., Dai, J., Shen, Y., Zhang, C., Hua, Q., Yang, X., & Zhang, L. (2022). Optimization of microbial cell factories for astaxanthin production: Biosynthesis and regulations, engineering strategies and fermentation optimization strategies. Synthetic and Systems Biotechnology, 7, 689-704. https://doi.org/10.1016/j.synbio.2022.02.001

Rodríguez-Sáiz, M., de la Fuente, J. L., & Barredo, J. L. (2010). Xanthophyllomyces dendrorhous for the industrial production of astaxanthin. Appl Microbiol Biotechnol, 88, 645-58. https://doi.org/10.1007/s00253-010-2814-x

Mehta, N., S, J., Kumar, P., Verma, A. K., Umaraw, P., Khatkar, S. K., Khatkar, A. B., Pathak, D., Kaka, U., & Sazili, A. Q. (2022). Ultrasound-Assisted Extraction and the Encapsulation of Bioactive Components for Food Applications. Foods, 11(19), 2973. https://doi.org/10.3390/foods11192973

Bello, U., Adamu, H., Amran, N. A., & Qamar, M. (2025). Green extraction technologies: process systems, techno-economic and lifecycle analyses. Cleaner Engineering and Technology, 100881.

https://doi.org/10.1016/j.clet.2025.100881

Rajendran, P., Somasundaram, P., & Dufossé, L. (2023). Microbial pigments: Eco-friendly extraction techniques and some industrial applications. Journal of Molecular Structure, 1290, 135958. https://doi.org/10.1016/j.molstruc.2023.135958

Zou, T. B., Jia, Q., Li, H. W., Wang, C. X., & Wu, H. F. (2013). Response surface methodology for ultrasound-assisted extraction of astaxanthin from Haematococcus pluvialis. Mar Drugs, 11(5), 1644-1655. https://doi.org/10.3390/md11051644

Chemat, F., Abert Vian, M., Ravi, H. K., Khadhraoui, B., Hilali, S., Perino, S., & Tixier, A. F. (2019). Review of Alternative Solvents for Green Extraction of Food and Natural Products: Panorama, Principles, Applications and Prospects. Molecules, 24(16), 3007. https://doi.org/10.3390/molecules24163007

Abolore, R. S., Jaiswal, S., & Jaiswal, A. K. (2024). Green and sustainable pretreatment methods for cellulose extraction from lignocellulosic biomass and its applications: A review. Carbohydrate Polymer Technologies and Applications, 7, 100396. https://doi.org/10.1016/j.cptech.2024.100396

Harith, Z. T., de Andrade Lima, M., Charalampopoulos, D., & Chatzifragkou, A. (2020). Optimised Production and Extraction of Astaxanthin from the Yeast Xanthophyllomyces dendrorhous. Microorganisms, 8(3), 430. https://doi.org/10.3390/microorganisms8030430

de la Fuente, J. L., Rodríguez-Sáiz, M., Schleissner, C., Díez, B., Peiro, E.,

& Barredo, J. L. (2010). High-titer production of astaxanthin by the semi-industrial fermentation of Xanthophyllomyces dendrorhous. Journal of Biotechnology, 148(3), 144-146. https://doi.org/10.1016/j.jbiotec.2010.05.018

Bey, H., Gtari, W., Aschi, A., & Othman, T. (2016). Structure and properties of native and unfolded lysing enzyme from T. harzianum: Chemical and pH denaturation. Int J Biol Macromol, 92, 860-866. https://doi.org/10.1016/j.ijbiomac.2016.08.006

Murashima, K., Kosugi, A., & Doi, R. H. (2003). Solubilization of cellulosomal cellulases by fusion with cellulose-binding domain of noncellulosomal cellulase engd from Clostridium cellulovorans. Proteins, 50(4), 620-628. https://doi.org/10.1002/prot.10310

Zhao, X., Zhang, X., Liu, H., Zhu, H., & Zhu, Y. (2019). Enzyme-assisted extraction of astaxanthin from Haematococcus pluvialis and its stability and antioxidant activity. Food Sci Biotechnol, 28(6), 1637-1647. https://doi.org/10.1007/s10068-019-00659-4

Granato, D., Barba, F. J., Kovačević, D. B., Lorenzo, J. M., Cruz, A. G., & Putnik, P. (2020). Functional Foods: Product Development, Technological Trends, Efficacy Testing, and Safety. Annual Review of Food Science and Technology, 11, 93-118. https://doi.org/10.1146/annurev-food-032519-051708

Storebakken, T., Sørensen, M., Bjerkeng, B., Harris, J., Monahan, P., & Hiu, S. (2004). Stability of astaxanthin from red yeast, Xanthophyllomyces dendrorhous, during feed processing: effects of enzymatic cell wall disruption and extrusion temperature. Aquaculture, 231(1-4), 489-500. https://doi.org/10.1016/j.aquaculture.2003.07.007

ABSTRACT IN KOREAN (국문 요약)

식품 산업에서 노령화 및 신종 감염병의 등장으로 인해 항산화 및 면역 증진을 위한 건강기능식품에 대한 관심과 소비가 크게 증가하고 있습니다. 이에 따라 기능성 화합물에 대한 연구가 활발히 수행되고 있습니다. 케토카로티노이드(ketocarotenoid) 계열의 붉은 색소인 아스타잔틴(astaxanthin)은 다른 카로티노이드(carotenoid)에 비해 약 10배 강한 강력한 항산화 특성으로 인해 연구되어 왔습니다. 그러나 아스타잔틴의 화학적 형태는 천연 추출물에 비해 생체 이용률(bioavailability) 및 안정성(stability)이 낮은 단점이 있습니다. 본 연구에서는 *Xanthophyllomyces dendrorhous*의 세포벽을 분해하는 셀룰라아제(cellulase) 효소를 재조합 미생물을 이용하여 개발하였습니다. 추가적으로 X. dendrorhous로부터 추출된 아스타잔틴을 HPLC를 이용하여 분석하였습니다. 그 결과, 재조합 미생물에서 효소 생산이 성공적으로 이루어졌으며, 제조된 효소의 효소 활성은 정성적 및 정량적으로 모두 확인되었습니다. 아스타잔틴 농도가 0.04966 mg/mL로 확인된 효소 반응 방법은 0.00251 mg/mL로 더 낮은 농도를 보인 대두유 혼합 방법에 비해 더 적합한 추출 방법으로 확인되었습니다. 이러한 결과를 통해 효소 반응을 이용한 추출 방법은 *X. dendrorhous*로부터 추출된 아스타잔틴을 함유하는 기능성 식품 시장에 직접 적용 가능할 것으로 기대됩니다.