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Development of *Aurantiochytrium limacinum* SR21 cultivation using salt-rich waste feedstock for docosahexaenoic acid production and application of natural colourant in food product

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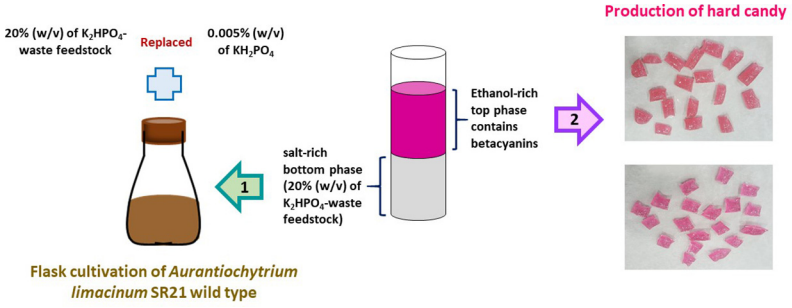
GRAPHICAL ABSTRACT



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ABSTRACT

Microalgae biorefinery is presently receiving a lot of attention as driven by its production of high value-added products. In this study, an oleaginous microalga *Aurantiochytrium limacinum* SR21 was cultured for docosahexaenoic acid (DHA) production using 20% (w/v) of K_2HPO_4-waste feedstock to replace 0.005% (w/v) of KH_2PO_4 in the flask culture. DHA is an essential nutrient for human's brain functionalities. Collectively, the K_2HPO_4-waste feedstock with working concentration of 0.005% (w/v) in the cultivation prompted a higher lipid content (8.29%) and DHA production (128.81 $mg.L^{-1}$). Moreover, natural plant pigment products containing stabilised betacyanins were utilised as natural red colourants for hard candy production. This study develops microalgal cultivation using salt-rich waste feedstock for a higher lipid and DHA content as well as application of natural colouring agents in food products.

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1. Introduction

Microalgal biotechnology applications are presently a rapidly growing field. In particular, microalgae biorefinery is gaining global interest as an emerging biomass transformation approach. Generation of high value-added products in addition to biofuel through microalgae biorefinery have been developed. For instance, microalgae biomass can be transformed into pigments, proteins, lipids, polyunsaturated fatty acids (PUFAs), carbohydrates, vitamins and antioxidants (Phong et al., 2017; Yen et al., 2013). These high value-added materials are subsequently applied in various commercial and industrial applications, such as foods, cosmetics, pharmaceuticals and nutraceuticals (Chew et al., 2017; Pulz and Gross, 2004; Spolaore et al., 2006; Wang et al., 2017; Yu et al., 2017). One of the major benefits of culturing microalgae is that they can accumulate high percentages of lipid in their bodies (approximately 20–50% of their total weight) (Brennan and Owende, 2010). Among the microalgal lipids is a long chain PUFA called docosahexaenoic acid (DHA) which play a vital role as health food supplements (Borowitzka, 2013; Tan et al., 2016).

DHA (22:6n-3) is classified as an important lipid in the omega-3 family because it is an essential nutrient for neurological and cognitive functions in humans (Bradbury, 2011; Calderon and Kim, 2004; Kawakita et al., 2006; Kim, 2007). A DHA-rich diet formulation is required for infants as DHA improves the growth and functional development of the brain in infants. DHA consumption is also important in adults as it aids in maintaining normal brain functionalities. Moreover, sufficient DHA intake showed a preventive role in non-communicable diseases, such as diabetes, cardiovascular diseases, neurodegenerative diseases, heart diseases and cancers (Bazan et al., 2011; Hashimoto, 2018; Horrocks and Yeo, 1999; Huang et al., 2012; Stillwell and Wassall, 2003).

Aurantiochytrium limacinum SR21, or previously known as *Schizochytrium limacinum* SR21 (Yokoyama and Honda, 2007), is a highly oleaginous microalga strain and reported as an excellent DHA producer. In addition, *Aurantiochytrium* species is easy to cultivate with short cultivation time. Previous studies have reported that cultivation of *A. limacinum* SR21 under optimised culture conditions produced high concentrations of lipid, DHA and biomass. Usage of different carbon sources, such as glycerol, crude glycerol and glucose, have prompted for higher lipid accumulation and microalgal growth in the cultivation of *A. limacinum* SR21 (Chi et al., 2007; Ethier et al., 2011; Gao et al., 2013; Huang et al., 2012; Li et al., 2015; Lung et al., 2016).

In recent years, natural colourants are receiving a lot of attention both from the consumers and food industries, as driven by the arising of negative health impacts on the utilisation of artificial colourants for food applications. Consumers prefer more safer, nutritious and healthier food products in addition to their appealing and delightful appearance. Natural colouring agents not only safe to consume but also offering some healthy functional. They can be obtained from natural pigments, such as betalains, anthocyanins and carotenoids. Betalains, especially betacyanins, are of growing interest due to their pH stability ranging from 3 to 7 which enable wide applications in colouring low acid to neutral foodstuffs (Carocho et al., 2015; Delgado-Vargas et al., 2000; Leong et al., 2018c; Martins et al., 2016; Moreno et al., 2008).

Taking the above into account, this study aimed to culture *A. limacinum* SR21 wild type (WT) using salt-rich waste feedstock, in addition to the use of glycerol as carbon source for DHA production. *A. limacinum* SR21 is known to be a microalga species with high lipid and DHA content. 20% (w/v) of K_2HPO_4-waste feedstock was used as a replacement ingredient for 0.005% (w/v) of KH_2PO_4 in the flask cultivation. The KH_2PO_4 and K_2HPO_4-waste feedstock with different number of moles of phosphate ions (PO_4^{3-}) were first evaluated, followed by working concentration of the salts. Analyses of microalgal growth and lipid production, in particular DHA, were assessed. Subsequently, natural plant pigment products containing stabilised betacyanins (Leong et al., 2018b) were utilised as natural red colourants for hard candy production. This study renews attention towards the potentiality of microalgae biorefinery in respect to economic and environment evaluations as well as sustainable management on the salt-rich waste feedstock. Also, natural colouring agents were applied in the food products.

2. Materials and methods

2.1. Materials

Microalga strain *A. limacinum* SR21 WT was kindly provided by the Biorefinery and Bioprocess Engineering Laboratory, Yuan Ze University, Taoyuan, Taiwan. 20% (w/v) of K_2HPO_4-waste feedstock was obtained from our previously conducted experiment using liquid biphasic system (Leong et al., 2019; Leong et al., 2018a). Natural colouring agents (peel and flesh extract of red-purple pitaya) were obtained from our previously performed experiment (Leong et al., 2018b). Food grade ethanol (99.8% (v/v)) was purchased from R&M Chemicals (Selangor, Malaysia). Other chemicals used in this experiment were of analytical grade (AG).

2.2. Flask cultivation of A. Limacinum SR21 WT

The microalga was first pre-cultured in a shaking incubator (TLT-806080, Cherny Huei Co. Ltd., Taiwan) for 3 days. This was then followed by transference of 10% (v/v) of inoculum from the pre-cultured stock into a 500 mL conical flask containing 90 mL of artificial seawater medium supplemented with 3% (w/v) of glycerol (total working volume was 100 mL). The culture medium was adjusted to pH 7.5. Subsequently, the microalga was incubated at 22 °C for 6 days at a shaking speed of 150 rpm with 12 h of illumination. The *A. limacinum* SR21 flourish under conditions of low temperature and mild alkaline due to better photosynthesis process (Lung et al., 2016).

The culture medium was prepared according to UTEX culture collection of algae with slight modifications (UTEX, 2018). It is an artificial seawater medium and composed of NaCl (18 $g.L^{-1}$), $MgSO_4{\cdot}7H_2O$ (2.6 $g.L^{-1}$), KCl (0.6 $g.L^{-1}$), $NaNO_3$ (1 $g.L^{-1}$), $CaCl_2{\cdot}2H_2O$ (0.3 $g.L^{-1}$), KH_2PO_4 (0.05 $g.L^{-1}$), tricine (4.48 $g.L^{-1}$; stock solution (224 $g.L^{-1}$) was adjusted to pH 8), NH_4Cl (0.027 $g.L^{-1}$), 10 $mL.L^{-1}$ of P-II metal solution and 1 $mL.L^{-1}$ of chelated iron solution. The P-II metal solution was composed of $Na_2EDTA{\cdot}2H_20$ (1 $g.L^{-1}$), H_3BO_3 (1.14 $g.L^{-1}$), $FeCl_3{\cdot}6H_2O$ (49 $mg.L^{-1}$), $MnSO_4{\cdot}H_2O$ (164 $mg.L^{-1}$), $ZnSO_4{\cdot}7H_2O$ (22 $mg.L^{-1}$) and $CoCl_2{\cdot}6H_2O$ (4.8 $mg.L^{-1}$). The chelated iron solution was prepared using 10 g of $Na_2EDTA{\cdot}2H_2O$ per 500 mL of distilled water and 0.81 g of $FeCl_3{\cdot}6H_2O$ per 450 mL of 0.1 M HCl; the total volume was brought up to 500 mL.

2.2.1. Replacement of KH_2PO_4 by K_2HPO_4-waste feedstock

The KH_2PO_4 in the culture medium (0.005% (w/v)) for *A. limacinum* SR21 WT cultivation was replaced by 20% (w/v) of K_2HPO_4-waste feedstock. The experiment was conducted using (1) different no. of moles of PO_4^{3-} (mol/L) and (2) similar working concentration (%, w/v) of the salts; the subsequent study with the chosen salt was further optimised using different working concentrations, in order to obtain an optimal result. In addition, 20% (w/v) of K_2HPO_4 salt (AG) was used to further confirm our experimental results obtained from the K_2HPO_4-waste feedstock.

2.3. Hard candy production

The peel and flesh extract of red–purple pitaya which play a role as natural red colourants were obtained from our previously performed experiment. As to produce hard candy, 100 g of sugar, 33 g of glucose syrup and 20 g of water were first mixed and boiled to 155 °C, followed by addition of some natural colouring and flavouring to the candy.