

Production of lipids containing high levels of docosahexaenoic acid from empty palm fruit bunches by *Aurantiochytrium* sp. KRS101

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Abstract The oleaginous microalga *Aurantiochytrium* sp. KRS101 was cultivated in enzymatic hydrolysates of alkali-pretreated empty palm fruit bunches (EFBs), without prior detoxification process. The maximal levels of lipid and docosahexaenoic acid synthesized were 12.5 and 5.4 g L⁻¹ after cultivation for 36 h. Similar lipid levels were also obtained via simultaneous saccharification and cultivation. The results suggested that EFB is a promising source for production of useful lipids by the microalgal strain.

Keywords Heterotrophic oleaginous microalga ·
Aurantiochytrium sp. · Docosahexaenoic acid ·
Lipid · Empty palm fruit bunch

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Introduction

Oleaginous microalgae of the Thraustochytrid family may be valuable sources of oil because at least 30 % of dry biomass is lipid, and they contain high levels of omega-3 polyunsaturated fatty acids (PUFAs) [1–3]. In particular, they are rich in docosahexaenoic acid (DHA, C22:6n-3), which plays key roles in brain and eye development [4–6]. Additionally, the high levels of desirable fatty acids render microbial oil valuable as a feedstock for production of biofuel [7].

Recently, we isolated and identified a novel Thraustochytrid microalga (termed *Aurantiochytrium* sp. KRS101) that produced high levels of lipid and DHA (over 45 % by weight of total fatty acids) [8]. Upon fed-batch fermentation in a 5-L bioreactor, good biomass levels and lipid production values (50 and 22 g L⁻¹, respectively) were obtained; DHA productivity (3.3 g L⁻¹ d⁻¹) was comparable to that of *Schizochytrium limacinum* SR21 [9]. The commercial potential of the microalgal strain was high, as revealed by successful cultivation in a pilot-scale bioreactor [10].

Many types of nutrients have been tested for the ability to support the growth of oleaginous microalgal strains. For example, Chi et al. [11] cultivated the Thraustochytrid microalga *S. limacinum* SR21 in a hydrolyzed potato broth. Liang et al. [9] employed sweet sorghum stalk juice for cultivation of a microalgal strain. Other feedstock for microbial lipids production was used such as corn cob hydrolysate [12], rice straw hydrolysate [13], and wastewaters [14]. In addition to agricultural products, crude glycerol, a byproduct of the biodiesel industry, has been used for cultivation of a microalgal strain. We earlier showed that molasses supported the growth of the microalga *Aurantiochytrium* sp. KRS101. In the present study,

we explored whether a cellulosic biomass would support growth of this microalgal strain and the production of lipids including DHA by the strain.

Materials and methods

Microalgal strain and media

Details of the Thraustochytrid microalgal strain *Aurantiochytrium* sp. KRS101 have previously been reported [8]. The microalga was cultivated in 500-mL baffled flasks containing 50 mL of basal medium [glucose 60 g L⁻¹, yeast extract 10 g L⁻¹, dried natural sea salt (CJ Co., Seoul, Korea) 10 g L⁻¹] at 28 °C, with shaking at 125 rpm, for 3 days.

Pretreatment and enzymatic saccharification of empty palm fruit bunches

Empty palm fruit bunches (EFBs) were supplied by Gendocs (Daejeon, Korea). EFB fibers were milled to a particle size of 1–2 mm, soaked in 1 M NaOH solution with shaking at ambient temperature for 30 min, autoclaved at 121 °C at 15 psi for 1 h, and finally washed with tap water to remove NaOH. EFB thus pretreated was mixed with a cellulase solution (Novozyme, Bagsvaerd, Denmark) to yield a mixture containing 10 % (w v⁻¹) solids at a concentration of 40 Filter Paper Assay Unit (FPU) per g of EFB; the mixture was incubated at 45 °C for 3 days with shaking at 150 rpm. The amount of PFEB added constituted 5–10 % of culture volume. Inoculum constituted 2, 5 and 10 % of culture volume. Treated enzyme was 40 FPU g⁻¹ of EFB. Glucose concentration of PFEB was 58 g L⁻¹. Cultured working volume was 600 mL in 2-L baffled flasks. Enzymatic hydrolysates of EFB were analyzed via HPLC (Young-Lin Instrument Co., Anyang, Korea); the instrument was equipped with a Waters 2410 refractive index detector (Waters, Milford, MA, USA), an autosampler, an analytical Rezex ROA-Organic Acid H+ column (7.8 × 300 mm; Phenomenex, Torrance, CA, USA), and a SecurityGard (KJ0-4282; Phenomenex). All samples were clarified by filtration through filters of 0.20- μ m pore size (DISMIC-13HP; Toyo Roshi Kaisha, Tokyo, Japan) prior to injection into the analytical column. The column temperature was at 65 °C. The mobile phase was 2.5 mM sulfuric acid at a flow rate 0.6 mL min⁻¹ under isocratic conditions. 5-Hydroxymethylfurfural (HMF) and furfural (Sigma-Aldrich, MO, USA) standard were analyzed via HPLC (Young-Lin Instrument Co.) by same detection method. Furfural standard solution (Sigma-Aldrich) was detected in concentration of 0.01, 0.02, 0.04, 0.08, 0.1 g L⁻¹ by HPLC. HMF (Sigma-Aldrich) was

dissolved in 2.5 mM sulfuric acid and then analyzed at concentration of about 0.01, 0.02, 0.04, 0.08, 0.1 g L⁻¹. The column temperature was at 65 °C. The mobile phase was 2.5 mM sulfuric acid at a flow rate 0.6 mL min⁻¹ under isocratic conditions.

Fermentation by *Aurantiochytrium* sp. KRS101

The enzymatic hydrolysate of pretreated EFB, without further detoxification, was used as a growth substrate for *Aurantiochytrium* sp. KRS101. Cells were precultured in basal medium, with shaking at 125 rpm, for 3 days at 28 °C, and then transferred at 2.5 % (v v⁻¹) to a 5-L fermenter containing fresh medium in which the hydrolysate was substituted for glucose. The fermentation conditions were 28 °C, stirring at 50 rpm, 0.5 v v⁻¹ min⁻¹ of air, and pH 7.0. Culture aliquots were collected every 12 h for analysis via the measurement of absorbance at 600 nm.

When simultaneous saccharification and fermentation (SSF) was to be performed, precultured cells were transferred into a 5-L fermenter in which the medium included pretreated EFB as carbon source and cellulose solution at 40 FPU g⁻¹ EFB.

Dry cell weight analysis

Dry cell weight (DCW) was estimated by harvesting cells at 4,500g at 4 °C for 20 min. Each supernatant was discarded and each pellet washed three times with phosphate-buffered saline (PBS, pH 7.2). Resuspended cells were again harvested by centrifugation at 4,500g at 4 °C for 20 min. Each pellet was resuspended in 600 μ L of distilled water and transferred to a preweighed vial. Cell pellets were dried at 60 °C for 12 h using a speed vacuum concentrator (Biotron 4080C; Bucheon, Korea). Each vial was weighed and DCW values were obtained.

Lipid analysis

Total lipid content was calculated using a modified (miniaturized) Bligh-Dyer method as described by Burja et al. [15]. Dried cells (125 mg) were placed in screw-cap test tubes, and 6.25 mL of chloroform, 12.5 mL of methanol, and 5 mL of 50 mM K₂HPO₄ buffer (pH 7.4) were added to each tube. Samples were agitated for 1 h at 200 rpm at 28 °C. Each sample was then transferred to a 50-mL graduated tube to which 6.25 mL of chloroform and 6.25 mL of phosphate buffer were added. Each tube was inverted 30 times and the phases allowed to separate for 1 h before the recovery of the bottom layer (approximately 12.5 mL). This liquid was transferred to a preweighed aluminum dish and the solvent evaporated over 30 min in a

dry oven at 80 °C. After cooling, the dish and contents were weighed, and total lipid levels were determined gravimetrically (to yield the weight of lipid extracted) using the following equation:

$$\text{Total lipid (g of oil per 100 g sample)} \\ = [(W_L - W_D) \times V_C \times 100] / [V_P \times W_S]$$

where W_D was the weight of an empty aluminum dish (g); W_L the weight of an aluminum dish with dried lipid residue (g); W_S the weight of sample (g); V_C the total volume of chloroform in the graduated cylinder (mL); and V_P the volume of chloroform transferred to the aluminum dish (mL).

Analysis of fatty acid composition

Dried cells were resuspended in 3 mL of 4 % ($v v^{-1}$) methanolic sulfuric acid and heated at 90 °C for 1 h in sealed vials. Fatty acid methyl esters (FAMES) were extracted into 0.3 mL of hexane and analyzed via gas chromatography (GC; Hewlett Packard 6890N; Ramsey, MN, USA); the instrument was equipped with a flame-ionization detector (FID) and an HP-5 (30 m \times 0.32 mm; 0.25 mm; Agilent Technologies; Santa Clara, CA, USA). The column temperature was raised from 150 °C (after 2 min of holding) to 270 °C (with a further 2 min of holding) at a rate of 7 °C per min.

Results and discussion

Cultivation of *Aurantiocytrium* sp. KRS101 in a medium containing enzymatic saccharification products of EFBs

Growth of *Aurantiocytrium* sp. KRS101 was first observed in a medium containing enzymatic saccharification products of EFBs; the composition of EFB enzymatic hydrolysate was 51.2 % of cellulose, 23.3 % of hemicellulose, 15.3 % of lignin and ash etc. The medium contained 58 g L⁻¹ of glucose and 18 g L⁻¹ of xylose (Fig. 1). As shown in Fig. 1, active growth of KRS101 was observed, and lipid production was noted; glucose was rapidly consumed. This indicated that no toxic materials such as HMF and furfural were present in the saccharification solution (data not shown). As expected, glucose was the first to be consumed; after this hexose was exhausted, the pentose xylose was next utilized. The maximal levels of lipid and DHA attained were 12.5 and 5.4 g L⁻¹, respectively. The composition of fatty acid of the oil produced was similar contents that; 5.1 % of myristic acid, 1.4 % of penta-decanoic acid, 36.6 % of palmitic acid, 1.9 % of EPA,

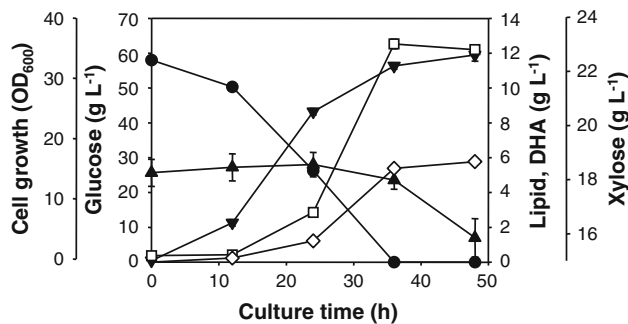


Fig. 1 Cultivation of *Aurantiocytrium* sp. KRS101 in a saccharified solution derived from empty palm fruit bunches (EFBs). Filled circles glucose levels, filled normal triangles xylose levels, filled inverted triangles cell growth, open squares lipid content, open diamonds DHA content. The error bar shows mean of the three independent analysis results. Standard deviation calculated from Student's *t* test. It represented the mean \pm SD

7.7 % of DPA ($n = 3$), 47.3 % of DHA ($n = 6$), respectively.

Although most of lignin, hemicelluloses and ash were washed off after pretreatment with NaOH, the remaining soluble lignin was at least 0.069 % of total PEFB, therefore it did not inhibit fruit bunch hydrolysis or downstream growth of algae. The carbon efficiency for DHA production based on total sugars was 10.2 % of the hydrolysate. The efficiency of hexoses versus pentose conversion into DHA was 95.8 versus 4.2 %. Also, artificial media containing the same sugar content as the hydrolysate produced similar 11.8 and 4.2 g L⁻¹ of lipid and DHA, respectively.

Simultaneous saccharification and fermentation (SSF) by *Aurantiocytrium* sp. KRS101 using pretreated EFB

Next, *Aurantiocytrium* sp. KRS101 was cultivated employing SSF using pretreated EFB. There are many advantages in utilizing SSF instead of separate hydrolysis and fermentation (SHF). For example, end-product inhibition of the enzymes in the enzymatic hydrolysis can be avoided, the risk of contamination is reduced (since the somewhat toxic hydrolysate after pretreatment tends to suppress bacterial growth), and capital investments are lower as the total reactor volume is decreased due to higher productivity.

When the medium contained 5 % ($w v^{-1}$) of pretreated EFB (final concentrations of 34 g L⁻¹ of glucose and 11 g L⁻¹ of xylose), most glucose was consumed in 3 days of cultivation, yielding 1.7 g L⁻¹ of lipid, and xylose was next utilized for further growth (Fig. 2a). The late stages of growth also featured the reuse of nutrient stored as lipid, thus decreasing lipid levels. To increase lipid production, the concentration of pretreated EFB was raised to 10 %

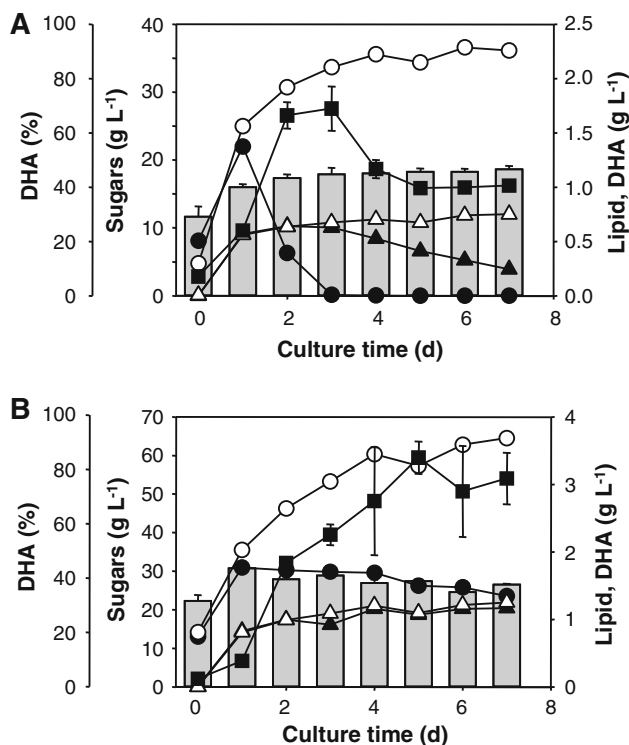


Fig. 2 Simultaneous saccharification and fermentation (SSF) by *Aurantiochytrium* sp. KRS101 using pretreated EFB. **a** EFB at 5 % ($w v^{-1}$); **b** EFB at 10 % ($w v^{-1}$). Filled circles glucose levels, filled triangles xylose levels, filled squares lipid content, gray bars DHA content, open circles and triangles glucose and xylose levels (in the absence of cell inoculation). The error bar shows mean of the three independent analysis results. Standard deviation calculated from Student's *t* test. It represented the mean \pm SD

($w v^{-1}$). Interestingly, the amount of glucose consumed ($31.1 g L^{-1}$) was similar to that metabolized in the above experiment; a large amount of glucose ($26.2 g L^{-1}$) remained in the culture solution upon cessation of glucose catabolism (Fig. 2b). Xylose was not consumed due to the effect of catabolite repression driven by residual glucose. However, the maximal level of lipid production increased

twofold ($3.4 g L^{-1}$). This enhanced production may be explained by the higher level of glucose in culture solution, which may have stimulated the synthesis of lipid as a stored nutrient. Although there is further scope for using corncorb, rice straw, corn stalk and sugarcane bagasses by H_2SO_4 hydrolysates or HCl hydrolysates (Table 1), this is the first article of lipid production by SSF.

Enhanced production of lipid on SSF by *Aurantiochytrium* sp. KRS101

In the SSF experiments described above, employing pretreated EFB at 10 % ($w v^{-1}$), it was likely that enzymatic saccharification was adequate; it was not necessary to add more cellulase. Therefore, the effect of another parameter, the inoculum level, on SSF was evaluated. As shown in Fig. 3a, utilization of sugars derived from EFB by enzymatic saccharification increased as the inoculum level rose from 2 to 10 % ($v v^{-1}$). The effect of inoculum level on lipid production was more notable. The maximal level of lipid production attained upon cultivation in SSF after inoculation at 10 % ($v v^{-1}$) was fourfold greater ($12.6 g L^{-1}$) than those noted when the inoculum levels were 2 and 5 % ($v v^{-1}$) (Fig. 3b). The productivity of lipid synthesis also increased by approximately threefold (from 0.68 to $1.8 g L^{-1} d^{-1}$) when the inoculum level was raised to 10 % ($v v^{-1}$). However, DHA contents were not notably affected by the inoculum level.

Conclusion

The heterotrophic microalga *Aurantiochytrium* sp. KRS101 produced lipids containing high levels of DHA upon simultaneous saccharification and cultivation and also upon growth in enzymatic hydrolysates of alkali-pretreated EFBs, even when the hydrolysates were not subjected to further detoxification. Further optimization of this

Table 1 Lipid accumulation efficiency from cellulosic materials by oleaginous microorganism

Carbon source	Treatment	Strain	Biomass ($g L^{-1}$)	Lipid content (%)	Lipid yield ($g L^{-1}$)	Reference
Corncorb	Enzymatic hydrolysates	<i>Trichosporon dermatis</i>	24.4	40.1	9.8	[12]
Corn stalk	H_2SO_4 hydrolysates	<i>Rhodotorula glutinis</i>	17.0	11.8	2.0	[16]
Rice straw	Enzymatic hydrolysates	<i>Chlorella pyrenoidosa</i>	2.8	56.3	1.1	[17]
Rice straw	H_2SO_4 hydrolysates	<i>Rhodotorula glutinis</i>	3.6	5.7	0.2	[16]
Rice straw	H_2SO_4 hydrolysates	<i>Trichosporon fermentans</i>	28.6	40.1	11.5	[13]
Sugarcane bagasses	HCl hydrolysates	<i>Yarrowia lipolytica</i>	11.4	58.5	6.7	[18]
Palm empty fruit bunches	Simultaneous enzymatic hydrolysis and fermentation	<i>Aurantiochytrium</i> sp. KRS101	34.4	36.3	12.5	This work

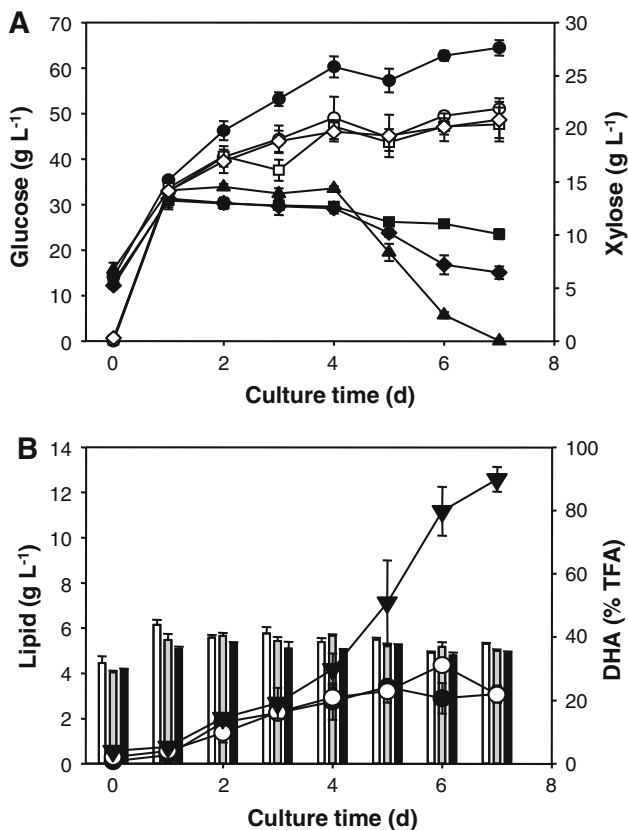


Fig. 3 Effect of inoculation size on SSF by *Aurantiochytrium* sp. KRS101. **a** Residual glucose (filled symbols) and xylose levels (open symbols). Inoculum size: circles 0 % ($v v^{-1}$), square 2 %, diamonds 5 %, open triangles 10 %. **b** Lipid content (open circles 2 %, filled circles 5 %, filled triangles 10 %) and DHA content (open bars 2 %, gray bars 5 %, filled bars 10 %). The error bar shows mean of the three independent analysis results. Standard deviation calculated from Student's *t* test. It represented the mean \pm SD

biological process may yield an economically viable method for industrial production of a valuable lipid from a byproduct of palm oil industry.

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