



Integrated production of long chain polyunsaturated fatty acids (PUFA)-rich *Schizochytrium* biomass using a nutrient supplemented marine aquaculture wastewater

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ABSTRACT

Schizochytrium limacinum SR21 was used to remove waste nutrients from a marine recirculating aquaculture wastewater while simultaneously producing a valuable biomass containing PUFA enriched with long chain fatty acids which may have potential as an aquafeed additive.

Clean seawater and marine aquaculture wastewater supplemented with yeast extract ($1.0\text{--}10\text{ g L}^{-1}$) and glycerol ($10\text{--}90\text{ g L}^{-1}$) were used for a comparative study of growth and PUFA production using *S. limacinum* in batch, continuous and in a packed bed reactor (pall rings, surface area $340\text{ m}^2/\text{m}^3$) that allow surface growth and cell recycling for a high cell density culture. Good growth rates ($\mu = 0.013\text{ h}^{-1}$) were obtained in batch and continuous culture modes. Biomass of *S. limacinum* was increased from 4.0 g L^{-1} as DCW in batch culture to 57.9 g L^{-1} in retention culture ($D = 0.062\text{ h}^{-1}$). Retention culture of *S. limacinum* in a packed bed reactor also produced total lipids of 36.5 g L^{-1} (53–65%, w/w as DCW) and long chain fatty acids ($>C_{22}$) of 12.6 g L^{-1} (21.8%, w/w as DCW) in 350 h. Long chain fatty acids of C_{22} , C_{18} and C_{16} , as the main fatty acids, were produced and production was enhanced when supplemented wastewater was used in growth medium. Changes to the relative concentrations of glycerol and yeast extract (C:N ratio) alter the amounts of oil produced and types of fatty acid produced.

S. limacinum in continuous culture mode ($D = 0.013\text{ h}^{-1}$) enabled significant reductions ($<90\%$) in phosphorous, nitrogen and ammonia concentrations of marine aquaculture wastewater and efficiency of wastewater treatment was further improved in retention culture operating at $D = 0.062\text{ h}^{-1}$. This system would be well suited to treating nutrient concentrated effluent from sludge sedimentation/dewatering tanks in recirculating aquaculture systems (RAS). The treatment capacity of the reactor was considerable and in this current study, 420 g N, 190 g P and 10 g total $\text{NH}_3\text{ day}^{-1}\text{ m}^{-3}$ reactor could be removed using the retention culture system.

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1. Introduction

Although recirculating aquaculture systems (RAS) represent an efficient use of water for growing fish, the wastewater released from aquaculture farms can contain high levels of nutrients, which are able to cause significant environmental impacts to receiving waters [Cripps and Bergheim, 2000; McIntosh and Fitzsimmons, 2003; Lovitt et al., 2008].

Recirculating aquaculture systems (RAS) commonly comprise of fish rearing tanks, pumping equipment for water circulation, equipment to remove insoluble particles by the gravitational and

mechanical methods (sedimentation tanks, sludge cone collectors, drum filters and protein skimmers) from the main stream; integrated biosystems (e.g. constructed wet lands, biofilter media) to remove or transform soluble organic and inorganic nutrients; and oxygen contactors for aeration [Losordo et al., 2000; Wik et al., 2009]. The principle waste product a watery sludge of collected insoluble particles is typically disposed of by spreading on the land directly as a fertilizer, mineralized in an anaerobic digester or put to landfill. The amounts of sludge and soluble nutrients discharged daily from recirculation aquaculture systems is dependent on species, feed, fish stocking density and type of wastewater treatment system employed (Table 1).

In recirculating aquaculture systems (RAS), the effluents are typically a stream of insoluble particles and suspending a nutrient-rich water which is passed through mechanical filters, biological filters or constructed wetlands to reduce the nutrient burden before being discharged or recycled [Lekang et al., 2000; Hunter et al., 2001;

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Table 1
Distributions of TSS, TN and TP in wastewater effluent from selected RAS according to daily feeds and species.

Species	Fish biomass (kg)	Storage (m ³)	T-Feed (kg)	TSS (kg)	TSS/feed (kg/kg)	TN/feed (kg/kg)	TP/feed (kg/kg)	Place	Ref.
<i>Salmo salar</i>	6071–14666	46–90	772–1720	41.2–59.5	0.063–0.194	0.03–0.077		USA, Commercial	Wolters et al. (2009)
<i>Oreochromis</i> sp.	1.5–13.5	0.64	2.99–4.23	0.27–0.33	0.078–0.09			Iran, Prototype	Raffee and Saad (2005)
<i>Oreochromis niloticus</i>	7.64–1076.4	20	204.6–519.3	71.3–349.2	0.35–0.67	0.026–0.031	0.015–0.018	USA, Commercial	Tworowska et al. (1997)
<i>Oxyeleotris marmorata</i>	0.6	0.195	Tilapia: 0.148	0.081	0.544	0.073		Malaysia, Prototype	Lam et al. (2008)
	0.6	0.195	Carp: 0.160	0.105	0.657	0.078			
	0.6	0.195	Scads: 0.155	0.120	0.777	0.082			
<i>Salvelinus alpinus</i>	15000–19500	150	3720–4650	930–1860	0.25–0.4	0.025–0.035		USA, Commercial	Summerfelt et al. (2004)
<i>Oncorhynchus mykiss</i>	51–431	10	232.6	46.9	0.2	0.006	0.007	Austria, Commercial	Partridge et al. (2006)
<i>Argyrosomus japonicus</i>	103.9–399	10	95.4	25	0.26	0.008	0.009	Austria, Commercial	Partridge et al. (2006)
<i>Lates calcarifer</i>	49.3–536	10	193.4	27.9	0.14	0.003	0.003	Austria, Commercial	Partridge et al. (2006)

Salmon, *Salmo salar*; Tilapia, *Oreochromis niloticus*; Marble goby, *Oxyeleotris marmorata*; Arctic char, *Salvelinus alpinus*; Rainbow trout, *Oncorhynchus mykiss*; Mulloway, *Argyrosomus japonicus*; Barramundi, *Lates calcarifer*.

Patterson et al., 2003]. Even if insoluble particles contain the majority of nutrients such as organic carbon, nitrogen and phosphorous, the dissolved nutrient content in wastewaters is still significantly elevated levels above ambient and can cause eutrophication of receiving waters or poor water quality within the fish farm [Barak and Rijn, 2000]. The effluent treatment methods such as bio-filtration (mainly for nitrification and denitrification), have been extensively investigated to improve both the quality of rearing water on the farm [Thomas et al., 2000; Sauthier et al., 1998; Rijn et al., 2006] and at the point of discharge [Hussenot et al., 1998; Klas et al., 2006].

Also, requirements process of wastewater and sludge treatment systems in fresh water and marine aquaculture farms differ, operating to specific standards and employing different biological and physical process to remove the nutrient burden in wastewater effluent. Marine effluents disposal is problematic requiring saline treatment processes that are predominately aerobic in nature, i.e. salt tolerant of organisms and the presence of the sulfate reduction in anaerobic systems, producing hydrogen sulfide [Aspé et al., 1997; Lefebvre and Moletta, 2006].

Likewise, the efficient wastewater treatment systems should be designed for the sustainability where expensive treatment may be offset by the production of a valuable product [Lemarié et al., 1998]. One approach is to utilize aquaculture wastewater as a nutrient source for the production of high value materials so that the wastewater can be cleaned while reducing the demand for nutrients that would otherwise be required for production of another organism.

Schizochytrium limacinum is a thraustochytrid that can grow in marine and estuarial environments. Many investigations concerning its physiology and biochemistry have been made and key feature of *S. limacinum* culture is the heterotrophic production of poly-unsaturated fatty acids, particularly Omega-3 fatty acids such as docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) [Guo et al., 1999; Tonon et al., 2002; Ward and Singh, 2005; Burja et al., 2006; Morita et al., 2006]. DHA-rich PUFA from *S. limacinum* have a potential to use for human and animal nutrition [Cardozo et al., 2007]. Researchers have investigated the safety of these products including studies on animal toxicity [Hammond et al., 2001a,b,c; Abril et al., 2003], nutrition [Hammond et al., 2001a,b,c], mutagenicity [Hammond et al., 2002] and reproduction [Hammond et al., 2001a,b,c]. PUFA-rich marine algae and *S. limacinum* can be used as aquaculture feeds. When marine microalgae and *S. limacinum* of dry powder form were fed, juvenile mussels grew rapidly [Langdon and Önal, 1999]. Feeding on fish with rotifer or *Artemia nauplii* enriched with marine single cell heterotrophs also resulted in enhanced growth of Atlantic halibut larvae and recorded a significant improvement on the differentiation rate of sea bass larvae [Harel et al., 2002].

S. limacinum accumulates PUFA in lipid bodies using glucose, glycerol or ammonium acetate as a carbon source for heterotrophic growth [Yokochi et al., 1998]. *S. limacinum* was investigated for production of long chain fatty acids and Xanthophylls in Shochu distillery wastewater in Japan [Yamasaki et al., 2006]. Three major fatty acids were found: docosahexaenoic acid (22:6n-3, DHA), docosapentaenoic acid (22:5n-6, DPA) and palmitic acid (C₁₆). The composition of fatty acids in *Schizochytrium* sp. has been shown to vary according to culture temperature and salinity [Zhu et al., 2007]. Also, the culture conditions of *S. limacinum* have been investigated for growth and production of PUFA in a wide range of carbon and nitrogen sources, and pH [Wu et al., 2005]. Furthermore, *S. limacinum* is of marine-origin and requires high levels of sodium for growth, providing a good opportunity to culture on marine aquaculture wastewaters.

In this study, the growth of *S. limacinum* SR21 was investigated for the simultaneous production of PUFA-rich biomass as a potential raw material for aquafeed supplements, while reducing the

levels of nitrogen, phosphorous and ammonia in waste effluents from an experimental marine RAS.

We report the growth rate and production of lipids and long chain fatty acids by *S. limacinum* in sterilized marine aquaculture wastewater supplemented with yeast extract and glycerol. The development of a cell retention culture mode was investigated for high density culture of *S. limacinum* to facilitate cell recovery from the process and to improve the efficiency of wastewater treatment.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased at general grade from Fisher scientific (U.K.). All fatty acids produced were identified by G.C using Standards of fatty acid methyl esters (FAME) of unsaturated (ME14-1KT) and saturated (ME10-1KT), as the references, purchased from Supelco (USA).

Marine aquaculture wastewater and clean seawater were obtained from the Centre for Sustainable Aquaculture Research (CSAR) at Swansea University. In CSAR the clean seawater supply had been subjected to sand filtration, ozonation, de-ozonation via activated carbon, followed by UV disinfection prior to use. The wastewater was obtained from an effluent sedimentation tank serving two 60 m³ marine recirculating aquaculture systems (RAS) stocked with various aquaculture species including European sea bass, Atlantic cod, European turbot and Pacific white shrimp. Water was recirculated to the livestock rearing tanks every 30 min to 1 h following purification by mechanical filtration, ozonated protein skimming, biofiltration and UV disinfection. Approximately 3% of each RAS by volume was replenished daily. The slurry of insoluble particles washed from the mechanical filters and protein skimmers was stored in a sedimentation tank, from which supernatant was pumped for use in the current trials. The slurry of insoluble particles in the sedimentation tanks will also spontaneously deteriorate under the influence of biological and chemical processes releasing soluble nutrients. Both the aquaculture wastewater pumped up from the sedimentation tanks and the clean seawater was used for formulation of *S. limacinum* growth media without further filtration.

2.2. Microorganism and propagation

Marine water was used in all culture media. Growth media for all cultures used in this investigation were sterilized by autoclave at 121 °C for 20 min before cultivation. The salinity of seawater used for cultivation was at 2.9–3.0% (w/v). Both seawaters used for culture media were supplemented with yeast extracts and glycerol otherwise particularly mentioned, even if the concentrations are different from one after another according to purpose of the experiment.

S. limacinum SR21 [Yokochi et al., 1998] was purchased from ATCC (MYA-1381TM). *S. limacinum* was grown at the shaking flask (120 rpm, Innova 44, New Brunswick Scientific) of 250 mL containing 100 mL sterile clean seawater supplemented with yeast extract (1.0 g L⁻¹) and glycerol (10 g L⁻¹) for 3 days (25 °C). Cells were harvested by centrifugation in sterile conditions and washed with 5 mL sterile clean water. After another centrifugation, pellets were collected and re-suspended in sterile clean water containing glycerol (25%, w/v). Vial suspensions were preserved at -70 °C until needed for inoculation.

2.3. Culture conditions

Vial suspensions were thawed at 30 °C immediately after taking out of -70 °C before inoculation. Cell suspensions were pre-

cultured in the 250 mL flasks containing culture medium of 100 mL seawater supplemented with yeast extract (1.0 g L⁻¹) and glycerol (10 g L⁻¹) for 3 days and then the pre-culture was transferred into main culture with inoculation volume of 5% (v/v) main culture.

Flask cultures were used for initial investigations of culture conditions and to inoculate the subsequent batch, continuous and retention cultures. Shake flask cultures, the main culture, were carried out in 250 mL Erlenmeyer flasks with working volume of 50 mL for 10 days. Glycerol was compared with glucose as a carbon source to support growth of *S. limacinum* in flask culture containing clean seawater and wastewater supplemented with 1.0 g L⁻¹ of yeast extract. The concentration of carbon sources was increased from nil to 150 g L⁻¹ to investigate the effect of carbon sources in total biomass and growth rate. Also, the effect of growth temperature in growth rate was investigated in flask culture containing clean seawater supplemented with yeast extract of 1.0 g L⁻¹ and glycerol of 10 g L⁻¹. The investigation was undertaken in the constant temperature chambers at 20 and 25 °C.

The bioreactor for batch culture (5.0 L) was a Pyrex-glass vessel fitted with a 100 mm rubber head plate through which a sample line, a pH probe and feeding lines for liquids and gases. The wastewater from CSAR was used for formulation of growth medium during the batch culture (Fig. 3). The cultures were agitated by a magnetic stirrer with aeration during the fermentation. The assembled reactor together with media was autoclaved prior to inoculation. After sterilization, the bioreactor was placed in constant temperature chamber at 25 °C. The pH was controlled by manually adding alkaline solution (NaOH 1.0 M) under sterile conditions whenever pH dropped below 6.0. A 1 L flask culture was used for inoculation of batch culture with 5.0% culture volume. The concentrations of yeast extract and glycerol were changed according to purpose of the investigation in the flask and the batch culture.

The bioreactor for the continuous culture had additionally a gravity weir fitted and was autoclaved assembled before being placed in a constant temperature chamber of 25 °C. For the continuous culture, *S. limacinum* was grown in the starter culture with marine aquaculture wastewater supplemented with yeast extract at 3.0 g L⁻¹ and glycerol at 50 g L⁻¹ for 165 h as a batch prior to the start of feeding. The continuous culture was fed with marine aquaculture wastewater supplemented with yeast extract at 1.0 g L⁻¹ and glycerol at 10 g L⁻¹. Dilution rate was controlled between 0.0062 and 0.013 h⁻¹ during the continuous culture.

2.4. Retention culture of *S. limacinum*

The bioreactor used for batch culture of *S. limacinum* was modified for retention culture using a sedimentation vessel of 250 mL and packing elements of polyethylene plastic pall rings (Fig. 1).

The packed bed was formed using polyethylene pall rings of 16 mm diameter × 16 mm long (HAT International Ltd, UK), to provide the consistent surface with a surface area of $\approx 340 \text{ m}^2 \text{ m}^{-3}$ of packing elements and with voidage characteristics necessary for good mixing and mass transfer. Fig. 1 shows pall rings packing and illustrates how the packing elements retain biomass in the bioreactor during the fermentation. Fig. 1 also shows how the ancillary equipment was organized for cell retention culture of *S. limacinum*. Biomass of *S. limacinum* was retained in the bioreactor by the packing elements, from which large flocked materials were blocked off and were easily collected by a gravitational sedimentation in the sedimentation vessel. The settled biomass was pumped back to the bioreactor by the peristaltic pump. The volume of the packing elements in the bioreactor was increased from 2.0 L ($\approx 40 \text{ g L}^{-1}$) to 5.0 L ($\approx 100 \text{ g L}^{-1}$) during the trials to increase the surface area and consequently the amounts of biomass accumulated in the bioreactor.

For the high cell retention system, marine aquaculture wastewater supplemented with yeast extract of 10 g L⁻¹ and glycerol of

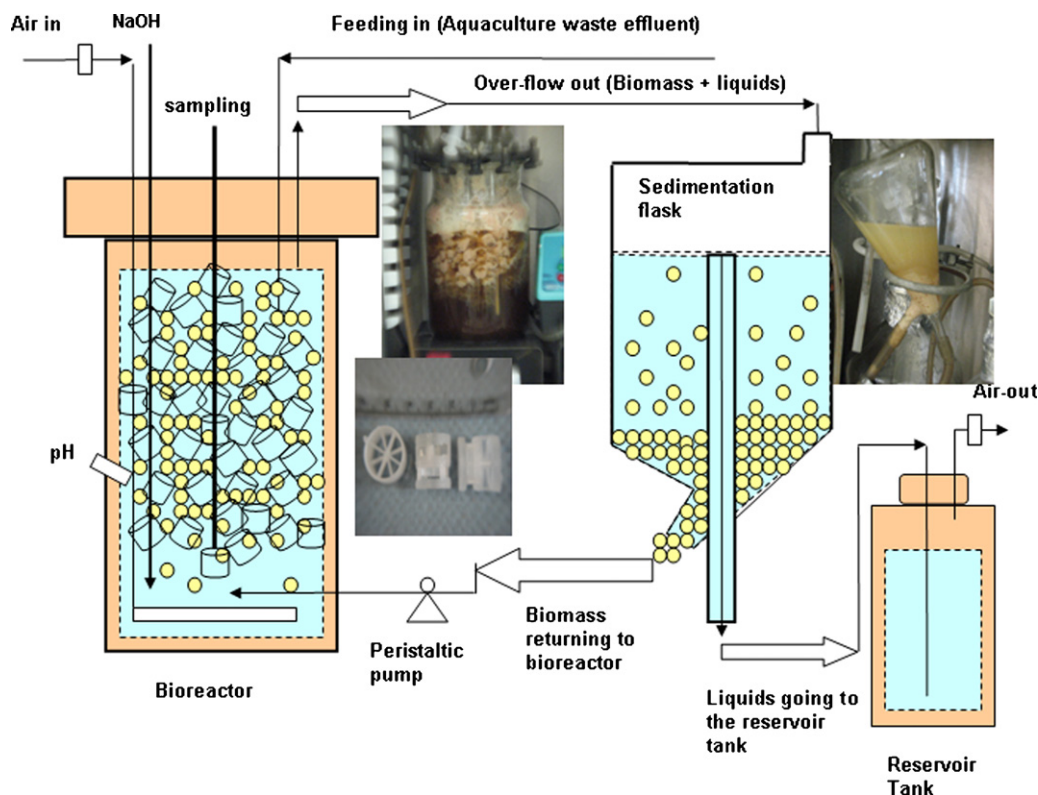


Fig. 1. Bioreactor (5.0 L) for the retention culture of *S. limacinum* SR21 constructed with the sedimentation vessel of 250 mL, the packing elements (5.0 L $\approx$ 500 g L⁻¹), the reservoir tank (5.0 L) and the peristaltic pump. Sedimentation vessel was placed between bioreactor and the reservoir tank. Packing elements (pall rings) as shown at the picture were randomly packed in the bioreactor. Biomass settled in the sedimentation vessel was pumped back to the bioreactor by the peristaltic pump on a daily basis. Only clarified liquid would pass into the effluent reservoir tank.

90 g L⁻¹ was used for the starter culture for 165 h prior to starting continuous feeding with marine aquaculture wastewater supplemented with yeast extract of 1.0 g L⁻¹ and glycerol of 3.0 g L⁻¹. Dilution rate was started at 0.013 h⁻¹, but when cell mass was increased, it was increased to 0.062 h⁻¹. Cell mass accumulating in the bioreactor was measured at the end of the investigation due to a difficulty in intermediate sampling (Table 2). The pH during retention culture was maintained above pH 6.0 by adding alkaline solution (NaOH, 1.0 M). The bioreactor was aerated via the air filter (Pall, U.K) at 1.0 L min⁻¹ and then ventilated through the effluent tank (5.0 L).

To investigate the effect of glycerol on total oil productivity the glycerol concentration in feed medium was changed from 10 g L⁻¹ to 90 g L⁻¹ using 1 g L⁻¹ yeast extract. The retention culture of *S. limacinum* used 80 g L⁻¹ packing materials.

2.5. Assay of cell mass

Cell growth in the culture was measured by absorbance at 550 nm 1 cm light path (Unicam 330 by Unicam Co. Ltd.) and was correlated to dry weight. Dry cell weight (g L⁻¹ as DCW) measurements were also made directly. Here the cultured broth (10 mL) was filtered through the filter paper (Whatman No. 2) and rinsed

to remove the salts in the filter paper with distilled water in three times. The washed filter papers were dried for 4 h at 105 °C. DCW was determined by a weight difference between the blank filter paper and the filtered.

2.6. Analysis of total nitrogen (TN), total phosphorous (TP) and total ammonia (TA)

For analyses of TP, TN and TA, the cultured broths were filtered through the glass filter (GF/C from Whatman). The permeates were subject to analyses of TP, TN and TA [Clescert et al., 1999]. TA (NH₃-N) was analyzed with phenol and alkaline hypochlorite to give indophenol blue in room temperature and developed blue colour is intensified with sodium nitroprusside. TP was measured as colourimetric determination of dissolved orthophosphates by vanadomolybdophosphoric acid method. TN was analyzed as NH₃-N after Kjeldhal digestion of the samples.

2.7. Analysis of glycerol, lipids and fatty acids

Glycerol was measured by HPLC (Varian) installed with the guard column and the analytical column (Carbopack MA1, Dionex),

Table 2
Production yields of long chain fatty acids, biomass and dilution rate in continuous culture and three different retention culture modes of *S. limacinum* SR21 ($n = 3$, SER; $\pm 3\%$).

Cultures	Culture time (h)	Dilution rate (h ⁻¹)	Biomass* (g L ⁻¹ , DCW)	Lipid content* (% w/w)
Continuous	600	0.013	4.30	53–65
Sedimentation	350	0.023	13.6	55.8–67.5
Sedimentation + packing (40 g L ⁻¹)	350	0.033	26.23	55.9–66.4
Sedimentation + packing (100 g L ⁻¹)	350	0.062	57.94	49.6–63.40

Packing elements of 40 g L⁻¹ is equal to 0.00045 m³ and 100 g L⁻¹ is equal to 0.001 m³.

* Biomass was measured as the amount accumulated in the bioreactor.

and the electrochemical detector (ED40, Dionex). NaOH (480 mM) was used in the isocratic mode for the mobile phase.

Samples were vigorously mixed with n-hexane (Hexane:sample=1:1) to remove lipids in the cultured broth and then the water and the lipid layer were separated in a micro-centrifuge for 3 min in 13,000 rpm. Only the water layer (middle) between pellet (bottom) and the lipid layer (upper) was injected into HPLC for glycerol assay.

For measurements of total lipids, the culture liquid was removed from the samples by centrifugation and then the pellets were washed with distilled water twice. Washed pellets were dried at 105 °C for 4 h. Lipids were extracted from dried biomass with a chloroform: methanol mixture in a 2:1 ratio until the colour of the solvent layer disappeared. The amounts of lipids (g of total lipids g⁻¹ as DCW) produced were determined by the weight difference between the blank flask and the flask containing the extracted oil after the solvent evaporation under vacuum.

Lipids were then subjected to acid methylation (Methanol-HCl in a 90 to 10 ratio) at 80 °C for 40 min. Fatty acid methyl esters (FAME) were extracted with n-hexane (Reaction mixture to hexane ratio was 1 to 1) for 1 h and then the n-hexane layer containing FAME was evaporated again under vacuum. FAME as a white powder was dissolved at dichloromethane and then injected into G.C.

The FAME were analyzed with G.C. (Varian 3800) fitted with Chrompack capillary column: Wcot fused silica (25 m × 0.32 mm ID coating CP-WAX 57CB, DF=0.2) from Varian and the rear FID detector (250 °C). The temperature range of column was from 75 °C to 250 °C. Temperature was maintained at 75 °C for 5 min and then increased to 140 °C at the rate of 14.0 °C min⁻¹ and increased again to 250 °C at the rate of 15 °C min⁻¹. Injector (Split type, 100%) was controlled at 250 °C.

3. Results

3.1. Water analysis

The aquaculture wastewater was found to contain TP, 8.33 ppm; TN, 4.42 ppm and TA, 2.82 ppm and the clean seawater was found to contain TP, 2.38 ppm; TN, 2.23 ppm and TA, 0.55 ppm (Table 4). Clearly, the marine wastewater contained significantly higher concentrations of dissolved inorganic nutrients than the clean seawater.

3.2. Cell growth in flask and batch cultures

Growth of *S. limacinum* was compared in 250 mL flask cultures (50 mL culture volume) containing clean seawater or aquaculture wastewater from the RAS effluent settlement tank (Fig. 2). The specific growth rate (μ) of *S. limacinum* was slightly lower in wastewater ($\mu = 0.011 \text{ h}^{-1}$) than clean water ($\mu = 0.012 \text{ h}^{-1}$) at 20 °C, but growth rate increased to 0.051 h⁻¹ in clean water and to 0.037 h⁻¹ in wastewater, respectively, as grown at 25 °C for 96 h. Cell mass in flask culture at which pH control was not applied for increased up to 3.0 g L⁻¹ (DCW) in clean water, but to 2.36 g L⁻¹ (DCW) in marine wastewater, and total cell mass did not change when the growth temperature was increased. The pH of the aquaculture wastewater dropped to 3.0 during the culture, whereas the pH of the clean water was maintained at pH 7.0. The reduced pH of the marine wastewater resulted in a reduction of cell mass at the end of flask culture (Fig. 2).

When glycerol was substituted for glucose as a carbon source, total cell mass did not show to be significantly different to the similar concentration of glucose. When the concentration of carbon sources was increased in the flask culture from nil to 70 g L⁻¹, total biomass increase to 2.8 g L⁻¹ using glycerol and 3.3 g L⁻¹ using glucose for 160 h, but there is no more increase when more carbon

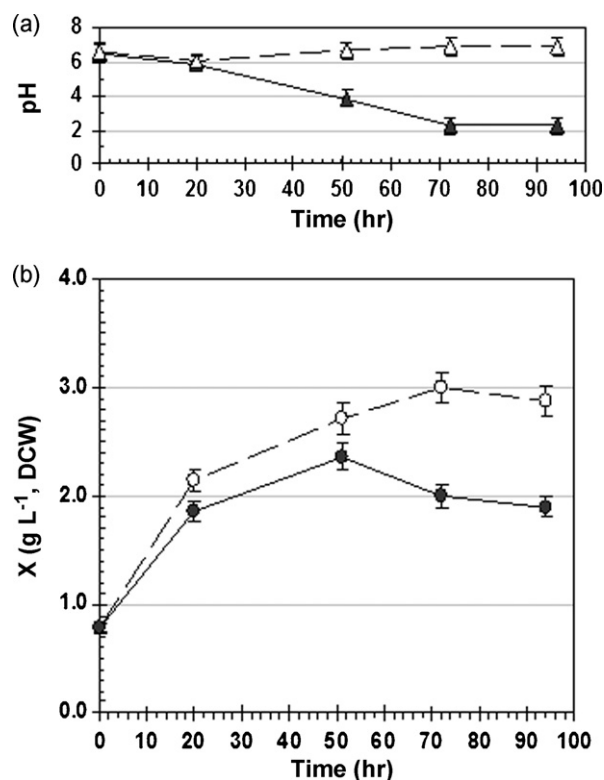


Fig. 2. Growth of *Schizochytrium limacinum* SR21 in time for flask culture ($n=3$, Standard error rate (SER); $\pm 3\%$). The pH (a) and growth (b) was compared at flask culture containing marine wastewater and clean water supplemented with yeast extract (1.0 g L⁻¹) and glycerol (30 g L⁻¹) during the culture of *S. limacinum*. Clean seawater (○); Wastewater (●).

source was added into flask culture. Cell growth was not detected in the absence of additions of a carbon source or yeast extract to the flask culture. Glycerol as a carbon source can be used at low cost in conjunction with the recycling process of biodiesel production [Chi et al., 2007]. Also, growth rate of *S. limacinum* was not affected by either type or the concentration of carbon sources in the flask culture.

Although the carbon sources did not affect total biomass of *S. limacinum*, yeast extract stimulated growth at a concentration up to 3 g L⁻¹, yielding an increase in specific growth rate to 0.037 h⁻¹ (from 0.005 h⁻¹). Specific growth rate did not increase further when yeast extract concentration was raised above 3 g L⁻¹ (data not shown).

When the flask culture of *S. limacinum* was scaled-up to 5.0 L of the batch culture using marine aquaculture wastewater supplemented with yeast extract of 3.0 g L⁻¹ and glycerol of 50 g L⁻¹, the specific growth rate decreased to 0.011 h⁻¹ from 0.037 h⁻¹, but total cell mass increased to 3.72 g L⁻¹ (DCW) in the batch culture for 150 h (Fig. 3).

3.3. Cell growth in continuous culture

The batch culture of *S. limacinum* was developed into the continuous culture using marine aquaculture wastewater supplemented with yeast extract (1.0 g L⁻¹) and glycerol (10 g L⁻¹) as feed medium. Biomass was washed out at dilution rate (D) above 0.013 h⁻¹, but when dilution rate was controlled below 0.013 h⁻¹, biomass increased to 4.3 g L⁻¹ as DCW (Fig. 4). 26.2 L supplemented wastewater was treated during the continuous culture of 600 h and total biomass of 50.9 g as DCW. The overflow from the bioreactor was collected (Table 2). Cell density in the bioreactor was 4.3 g L⁻¹ in end of the continuous culture.

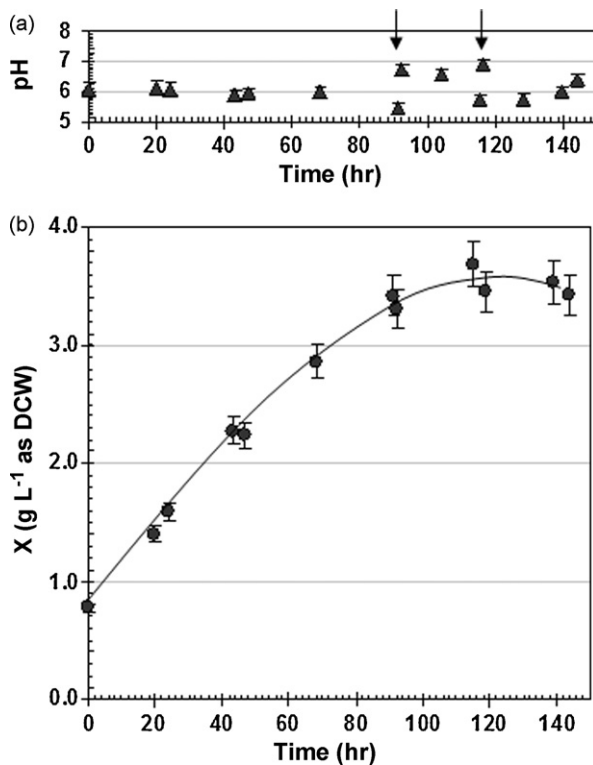


Fig. 3. Growth of *S. limacinum* SR21 in time for batch culture ($n=3$, SER; $\pm 3\%$). The batch culture was performed at marine aquaculture wastewater supplemented with yeast extract (3.0 g L^{-1}) and glycerol (50 g L^{-1}). The pH (a) and growth (b) are shown during the fermentation. NaOH (1.0 M) was manually added at 92 h and 115 h when pH went down below pH 6.0 ($\downarrow$).

3.4. Retention culture of *S. limacinum* in a packed bioreactor

A packed bed bioreactor for the cell retention culture was developed from the continuous culture to produce a high cell density

culture of *S. limacinum* by allowing surface growth on the plastic packing elements of 5.0 L ($\approx 100 \text{ g L}^{-1}$) and recycling of *S. limacinum* by the sedimentation vessel fitted into the bioreactor (Fig. 1). The retention culture of *S. limacinum* also improved the efficiency of wastewater treatment by increasing flow rates above the critical dilution rate ($D=0.013 \text{ h}^{-1}$) in the continuous culture, so allowing a consequent reduction in retention time and increased efficiency of the wastewater treatment.

In the bioreactor cell density of 4.3 g L^{-1} as DCW obtained at end of the continuous culture increased to 13.6 g L^{-1} as DCW during the retention culture with addition of the sedimentation vessel to allow cell recycling. When pall rings were used at a volume of 2.0 L ($\approx 40 \text{ g L}^{-1}$) in the bioreactor with the sedimentation vessel fitted, biomass in the bioreactor increased to 26.2 g L^{-1} as DCW for 350 h. The concentration of biomass accumulated in the bioreactor was further increased to 57.9 g L^{-1} over 350 h when the 5.0 L bioreactor was fully packed to 5.0 L ($\approx 100 \text{ g L}^{-1}$) with the pall rings (Table 2). Total aquaculture wastewater of 58.9 L was treated during the retention culture and the retention culture could produce *S. limacinum* biomass of 289.5 g for 350 h. Total biomass (289.5 g) was accumulated in the bioreactor by 13.5 and 5.7 times higher when compared to the batch culture (21.5 g) and the continuous culture (50.9 g), respectively.

It was possible to increase the dilution rate of marine aquaculture wastewater during the retention culture to 0.062 h^{-1} , which is 4.8 times higher than 0.013 h^{-1} in the continuous culture since biomass was retained by the pall rings in the bioreactor and recycled in the sedimentation vessel even in the high dilution rate of the supplemented wastewater (Table 2).

3.5. Production of total oil and poly-unsaturated fatty acids (PUFA) in culture of *S. limacinum*

Production of total oils and PUFA ($\text{g of PUFA per g of DCW}$) were compared when growing *S. limacinum* in the flask culture containing either clean seawater or marine aquaculture wastewater

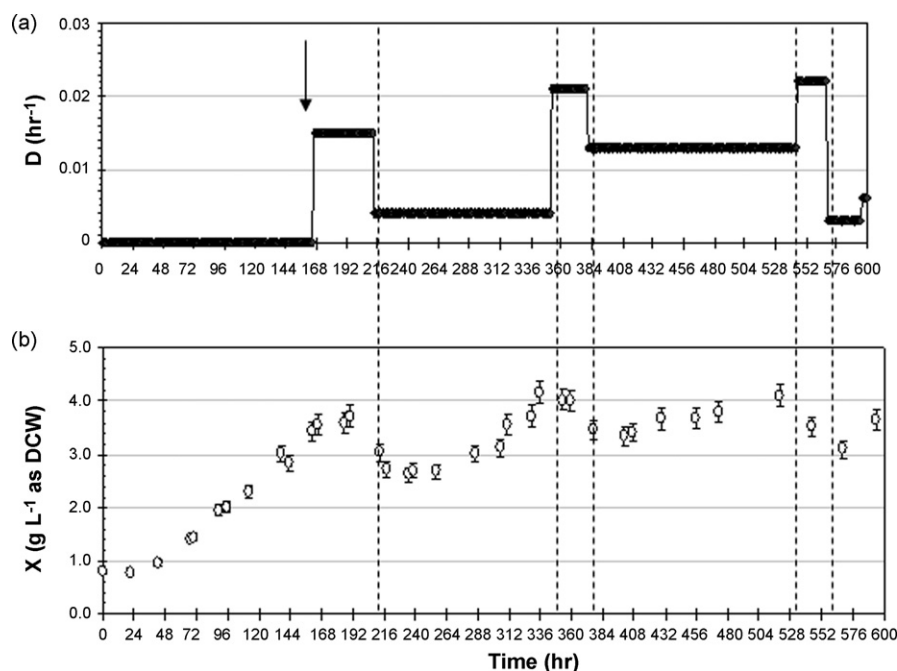


Fig. 4. Growth of *S. limacinum* SR21 in continuous culture Cell was grown in marine aquaculture wastewater supplemented with yeast extract (10 g L^{-1}) and glycerol (50 g L^{-1}) like batch culture, and then the culture of *S. limacinum* was fed with feed medium of marine aquaculture wastewater supplemented with yeast extract (1.0 g L^{-1}) and glycerol (10 g L^{-1}) during the continuous culture. Feeding was started at $D=0.015 \text{ h}^{-1}$ ($\downarrow$) and dilution rate was controlled in a certain range from 0.022 – 0.003 h^{-1} . The pH was controlled with NaOH (1.0 M) higher than pH 6.0 by manual addition. The experiment was undertaken in five times (SER; $\pm 2\%$).

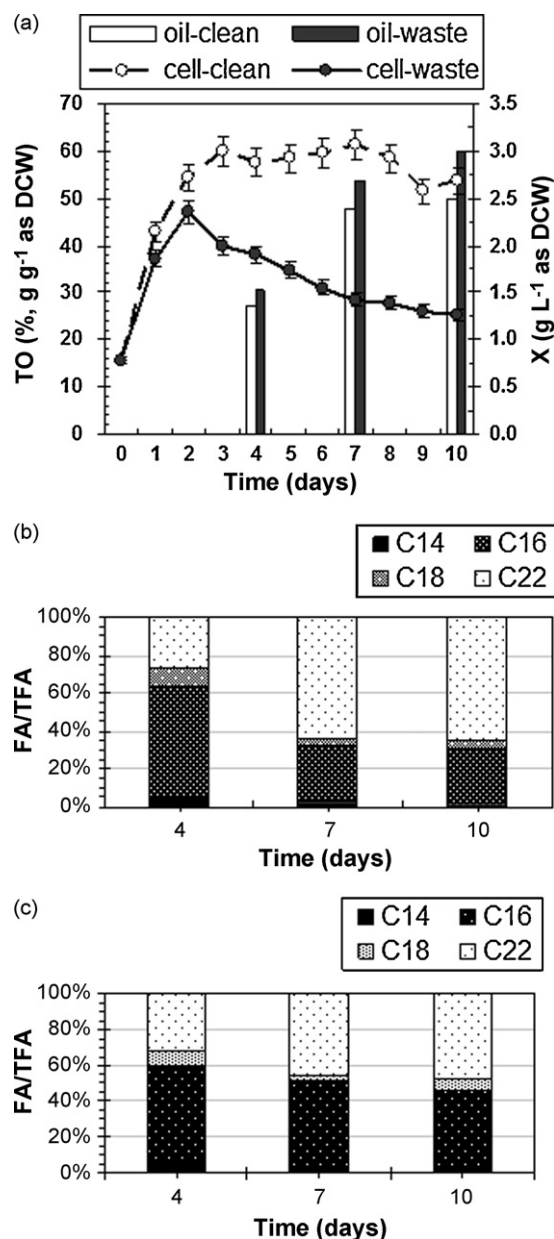


Fig. 5. The effect of culture age on total oils (TO) at supplemented growth medium with marine aquaculture wastewater and clean seawater during the culture of *S. limacinum* SR21 (SER; $\pm 5\%$). Production yields (% g of total oils g⁻¹ of DCW) of total oils were compared in flask culture using marine aquaculture wastewater (●) and clean seawater (○) supplemented with yeast extract 1.0 g L⁻¹ and glycerol 10 g L⁻¹ for 10 days. A variation on the composition of fatty acid during the culture of *S. limacinum* was displayed as the proportion of each fatty acid (FA) to total fatty acids (TFA). (a) Cell growth and production of total oils; (b) fatty acids in marine aquaculture wastewater; (c) fatty acids in clean seawater.

supplemented with yeast extract (1.0 g L⁻¹) and glycerol (30 g L⁻¹). Over the course of 10 days in the flask cultures, total oil content increased to almost 60% as DCW when using marine aquaculture wastewater, compared to 53% as DCW in clean seawater. Total oil production increased in both culture media with age, but more so in marine aquaculture wastewater than clean seawater after 7 days (Fig. 5). Oil production by *S. limacinum* on marine aquaculture wastewater required the effective control of the pH above 6.0 to maintain growth of biomass (Fig. 2). Fig. 5 also shows a difference in the composition of fatty acids produced during the culture of *S. limacinum* in supplemented marine aquaculture wastewater versus clean seawater. Long chain fatty acids (>C₂₂) increased to 62.38% of

Table 3

Variations in fatty acids with culture age during the continuous culture of *S. limacinum* SR21 (SER; $\pm 5\%$). Total oil was 53–63% as DCW during the continuous culture.

Time (h)	<i>D</i> (h ⁻¹)	C ₁₄	C ₁₆	C ₁₈	C ₂₂	Others
42–161	0	2.54	42.76	34.43	9.17	11.10
161–213	0.013	6.09	72.74	7.43	12.11	1.69
214–353	0.004	4.58	61.38	6.15	23.56	4.33
356–400	0.022	3.48	48.96	9.0	34.48	4.06
401–520	0.013	3.41	51.19	7.69	34.60	3.11

Portions of fatty acids were predicted as grams of fatty acids in gram of total lipids. *D*: Dilution rates (h⁻¹).

total fatty acids (TFA) in aquaculture wastewater with culture age, but only increased to 45.15% of TFA in clean seawater.

The maximum total oil contents of *S. limacinum* cultured continuously using marine aquaculture wastewater in 5.0 L vessels was the similar to that found in the flask cultures, at 53–65% as DCW (Table 2). Table 3 summarizes the time course of fatty acid composition during the continuous culture, illustrating a lower proportion of long chain fatty acids (>C₂₂) (20–30% of TFA) compared to that recorded in flasks (62% of TFA).

As illustrated in Fig. 5 and Table 3, short chain fatty acids (C₁₄–C₁₈) predominated, initially in both the flask and the continuous cultures, however the proportion of long chain fatty acids (>C₂₂) increased with simultaneous reduction of short chain fatty acids (C₁₄–C₁₈) with culture age. Total oil produced during the retention culture was 55–65% (w/w) of DCW without variation on total oil content produced during the scale-up of the cultures (Table 2).

The effect of glycerol concentration in total oil production by *S. limacinum* was investigated gradually increasing glycerol concentration in feed medium using 80 g L⁻¹ pall ring in a packed bed reactor. After initial inoculation on standard medium (30 g glycerol L⁻¹) the organism was grown in the batch culture until exhaustion of the glycerol carbon sources. At this point the system was switched to a continuous modes with different concentration of glycerol in the feed medium. The glycerol concentration in feed medium was increased while maintaining the yeast extract concentration at 1.0 g L⁻¹. At the end of the batch culture the oil content was 63.4 $\pm$ 8.02%; At steady using 10 g L⁻¹ glycerol the total oil content dropped to 47.6 $\pm$ 1.94%; using 30 g L⁻¹ glycerol 49.56 $\pm$ 0.81%, with 50 g L⁻¹ total oil content was 49.76 $\pm$ 1.48% and at 90 g glycerol L⁻¹ the total oil content was 41.15 $\pm$ 6.0%. This represents reduction by about 65.0% at total oil production over the range of glycerol used. At the same time, the long chain fatty acid contents of TFA (%) increased from 12.1 $\pm$ 0.52% at the end of the batch culture, 11.58 $\pm$ 0.43% using 10 g glycerol L⁻¹ feed, 14.46 $\pm$ 1.05% with 30 g glycerol L⁻¹ feed, 16.87 $\pm$ 0.81% with 50 g L⁻¹ and 22.92 $\pm$ 0.21% with 90 g glycerol L⁻¹. Therefore, as glycerol concentration increased in the retention culture using the aquaculture waste effluents, total oil productivity declined due to high glycerol concentration in the bioreactor, but long chain fatty acid content increased simultaneously.

3.6. Effects of different *S. limacinum* culture conditions on inorganic nutrient removal from marine aquaculture wastewater

During the exponential phase of the flask culture, TP and TN concentrations in yeast extract (3.0 g L⁻¹)-supplemented marine aquaculture wastewater decreased from initial values of 62.62 mg L⁻¹ and 190.3 mg L⁻¹, respectively to 15.4 mg L⁻¹ and 105.65 mg L⁻¹. TP reduced further to 10.28 mg L⁻¹ during the stationary phase of the flask culture; however, TN increased again to 575.2 mg L⁻¹ toward end of the culture. A similar trend of TP and TN reduction with culture age was observed in 5.0 L the batch cultures (data not shown), illustrating the importance of the growth phase to efficient nutrient removal.

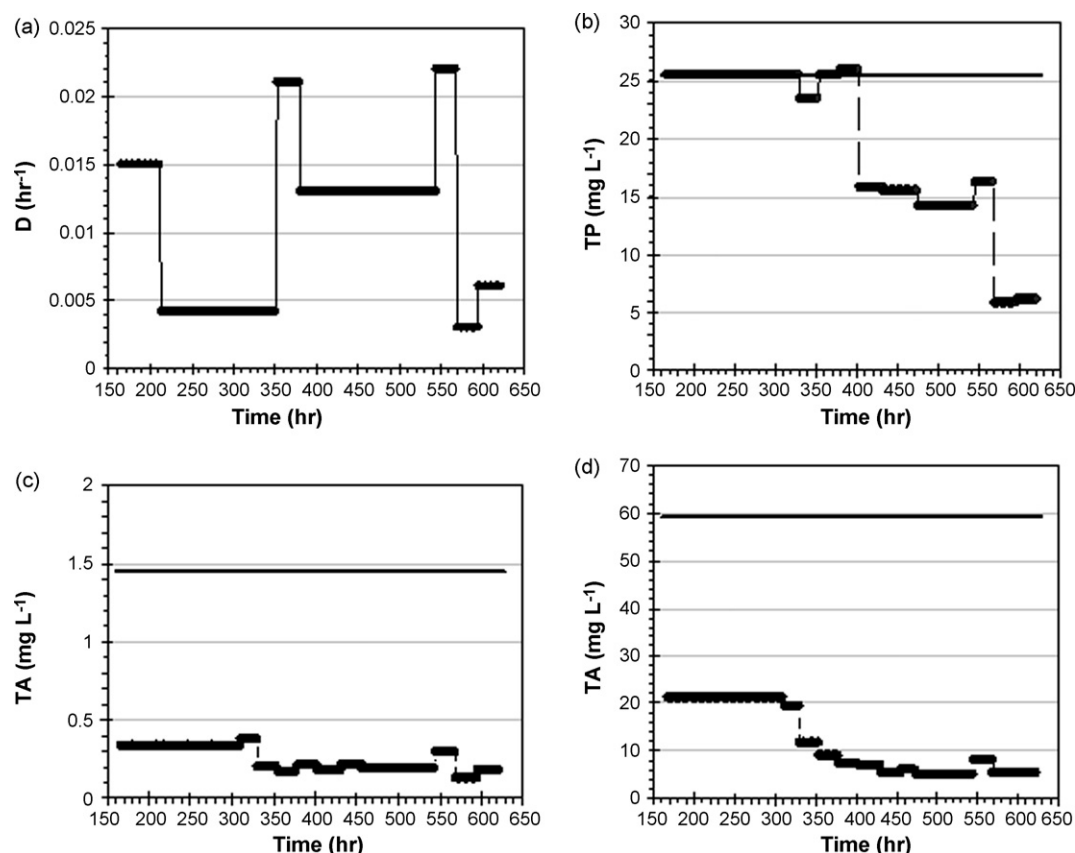


Fig. 6. The effect of continuous culture on the total phosphorous, total nitrogen and ammonia in the fluids before (●) and after (○) treatment ($n = 3$, SER; $\pm 5\%$).

The changes in TP, TN and TA during the continuous feeding of supplemented marine aquaculture wastewater are summarized in Fig. 6. Initial concentrations of TP, TA and TN were 25.46 mg L^{-1} , 1.45 mg L^{-1} and 58.9 mg L^{-1} , respectively as measured in marine wastewater supplemented with 1.0 g L^{-1} of yeast extract after filtration (GF/C from Whatman). In continuous mode using marine wastewater effluent supplemented with 1.0 g L^{-1} yeast extract and 30 g L^{-1} glycerol with *S. limacinum*, TN and TP levels were reduced by up to 77.3% and 91%, respectively of the initial levels in feed medium, while TA level was reduced by 91.0%.

The achievements of higher dilution rates during the retention culture of *S. limacinum* enabled greater volumes of marine aquaculture wastewater to be processed for inorganic nutrient removal. Table 4 summarizes TP, TN and TA levels in wastewater before and after treatment by *S. limacinum* culture in cell retention mode, illustrating great reduction (>90%) in TN and TA, and 78% reduction in TP. During the supplemented wastewater effluent treatment in cell retention mode of *S. limacinum*, treatment efficiencies were improved. Volumetric reduction rate of ammo-

nia ($R_{A/T}$) was improved by 2.1 times during the treatment in cell retention mode and volumetric reduction rate of phosphorous was improved by a 4.85 times. As the effect of cell retention mode, dilution rate (D) was improved by 4.8 times allowing more wastewater to treat in the same period (Table 5).

4. Discussion

S. limacinum has been the subject of many studies because it produces poly-unsaturated fatty acids (PUFA) for human foods and marine aquaculture feeds [Cardozo et al., 2007; Langdon and Önal, 1999; Harel et al., 2002]. Currently most Omega 3-rich PUFA produced by *Cryptocodinium cohnii* (DHASCO: Martek Columbia, MD, USA) and *Schizochytrium* sp. (Omega-tech, USA) are recently used with special emphasis of safety for human consumption [Spolaore et al., 2006], but PUFA rich biomass of two organisms also have been used for aquaculture feeds [Langdon and Önal, 1999; Harel et al., 2002; Spolaore et al., 2006; Cardozo et al., 2007; Miller et al., 2007; Ganuza et al., 2008a,b].

The use of nutrient-rich marine aquaculture wastewater to obtain *Schizochytrium* biomass with high lipid and high PUFA content offers an interesting, integrated approach to effluent remediation and valorization. Growing global aquaculture production is both increasing the demand for alternative (sustainable) feed ingredients [Li et al., 2004] and creating greater environmental impact via farm discharges [Cripps and Bergheim, 2000; McIntosh and Fitzsimmons, 2003; Lovitt et al., 2008].

Production of PUFA varies with growth conditions and composition of growth medium, growth rate, temperature, pH and the culture age [Fan et al., 2001; Wu et al., 2005; Zhu et al., 2007]. Table 6 is to compare biomass, lipid and DHA productions in fermentation of *Schizochytrium* sp. and *C. cohnii* using many different methods.

Table 4

Reduce of total phosphorous (TP) total nitrogen (TN) and total ammonia (TA) in marine aquaculture effluents during the retention culture of *S. limacinum* in packed bed reactor ($\approx 100 \text{ g L}^{-1}$ of pall rings) ($n = 2$, SER; $\pm 5\%$).

Nutrient	Clean water (g m^{-3})	Aquaculture wastewater (g m^{-3})	Treated wastewater (g m^{-3})
TP	2.38	8.33	1.89
TN	2.23	4.42	0.38
TA	0.55	2.82	0.26

Dilution rate was 0.062 h^{-1} using 5.0 L bioreactor and aquaculture waste water treatment was undertaken in 25°C for 350 h. As a result, except for 150 h for initiative propagation of *S. limacinum* prior to feeding of waste water, total 67.0 L aquaculture waste water was treated in 200 h.

Table 5

Efficiency of marine aquaculture wastewater treatment in cell retention mode and continuous mode of *S. limacinum* culture. Volume of the bioreactor was 5.0 L. Wastewater for feeding was supplemented with 1.0 g L⁻¹ yeast extract and 30 g L⁻¹ glycerol ($n = 2$, SER; $\pm 5\%$).

Culture	T	D	T-Vol	X	$R_{P/T}$ (P)	$R_{N/T}$	$R_{A/T}$ (NH ₃ -N)
C	450	0.013	70	4.3	0.039	0.194	0.005
R	200	0.062	67	57.9	0.190	0.421	0.010
Productivity ratio (R/C)		4.8		13.5	4.85	2.18	2.1

C, continuous mode; R, cell retention mode in packed bed reactor ($\cong 100$ g L⁻¹ of pall rings); T, operation time (h); D, dilution rate (h⁻¹); T-Vol, treated volume (L); X, biomass as DCW (kg m⁻³); $R_{P/T}$, volumetric reduction rate of phosphorus (kg day⁻¹, m⁻³); $R_{N/T}$, volumetric reduction rate of nitrogen (kg day⁻¹, m⁻³); $R_{A/T}$, volumetric reduction rate of ammonia (kg day⁻¹, m⁻³).

According to Table 6, Unagul et al. (2007) produced 28 g L⁻¹ DHA rich biomass of *S. mangrovei* in 108 h, using coconut water as a nitrogen source and Yamasaki et al. (2006) could produce 30 g L⁻¹ DHA rich biomass of *Schizochytrium* sp. in 120 h using Shochu distillery wastewater. In this investigation using a packed bed reactor for high cell density culture of *S. limacinum*, 57.9 g L⁻¹ total biomass, 36.7 g L⁻¹ total lipid and 12.7 g L⁻¹ long chain fatty acids (>C₂₂) were produced in 350 h using aquaculture waste effluent supplemented with glycerol 30 g L⁻¹ and yeast extract 1.0 g L⁻¹. Marine aquaculture wastewater enriched with a variety of nutrients affected the proportion of long chain fatty acids of TFA, and total oil productivity during the culture of *S. limacinum*. Additions of glycerol and yeast extract into the tested source of marine aquaculture wastewater, by providing a carbon source and increasing the concentrations of TN and TP to required levels, were found to be beneficial to long chain fatty acid-rich *Schizochytrium* biomass production. A crude glycerol, also as byproduct in transesterification of fatty acids to its methyl ester form which is a manufacturing process of biodiesel from vegetable oils, can be efficiently used as a cheap carbon source for growth of *S. limacinum* when compared with other carbon sources [Chi et al., 2007].

In this investigation, the production *S. limacinum* containing high total lipid and PUFA contents was shown to be possible, especially when using supplemented marine aquaculture wastewater, both improving the quality of wastewater and saving the use of the expensive nutrients such as urea, minerals, glucose, and corn steep liquor (CSL) required for growth of this organism [Yokochi et al., 1998; Shirasaka et al., 2005; Wu et al., 2005]. *S. limacinum* in the flask culture produced 10–12% more total oils (TO) as DCW in marine aquaculture wastewater than clean seawater, but growth rate reduced during the growth in wastewater. Also, a significant problem in using wastewater was a reduction in biomass production caused by the pH declined in end of fermentation in the flask culture (Fig. 2). Therefore, maintaining above 6.5 the pH of the batch culture was effective in increasing *Schizochytrium* biomass using wastewater, but growth rate did not increase.

In this work, the total oil productivity was found to be comparable among different culture systems, however the proportion of long chain fatty acids (>C₂₂) to total fatty acids (TFA) was sub-

stantially lower in 5.0 L continuous cultures than in the batch and the flask culture. To investigate the effect of glycerol concentration (and consequently C:N ratio) on total oil productivity using the wastewater in the retention culture, the glycerol concentration in feed medium was increased from 10 g L⁻¹ to 90 g L⁻¹ while maintaining 1.0 g L⁻¹ yeast extract. The results of this experiment showed that total oil contents decreased from 63.4% as DCW to 41.15% as DCW, but long chain fatty acid contents of TFA increased as a proportion from 12.1% to 22.92% of TFA indicating that the formulation of the wastewater medium has a critical effect on the quantity and the type of oils produced.

The growth in the flask cultures of *S. limacinum* could not detected unless additions of carbohydrates and yeast extract were made. Previous studies with *S. limacinum* have shown that the intracellular oil content and the proportion of long chain fatty acids to TFA could vary with C:N ratio in the culture medium. A lack of nitrogen source leads an increase of long chain fatty acid. Also mono carbohydrates, rather than poly carbohydrates help higher production of long chain fatty acids [Yokochi et al., 1998; Wu et al., 2005]. The nutrients in the wastewater used for this investigation was a relatively aged slurry in the sedimentation tanks and that maturation is microbiologically mediated and would reduced the levels of readily available carbohydrates. The higher total oil productivity in wastewater could be attributed to such a poor growth medium derived from soluble materials in sedimentation tank water is low in utilizable carbohydrate. However, when a good carbon source such as glycerol was supplied for growth, total oil production was reduced, but long chain fatty acid fraction of TFA increased.

S. limacinum is relatively slow grower when compared to growth rate of other industrial strains and easily washed out in marine wastewater during the continuous culture ($\mu_{\max} = 0.013$ h⁻¹). A high cell retention culture including an addition of yeast extract as a growth stimulator was beneficial in enabling a more rapid treatment of supplemented marine wastewater through the bioreactor, while simultaneously increasing the concentration of *S. limacinum* as a biocatalyst (Table 5). The cell retention mode used in this investigation showed a potential to treat waste effluents or sludge water from aquaculture farms prior to discharging or recycling.

Table 6

Comparison of biomass, lipid and DHA productions in *Schizochytrium* sp. and *Cryptocodinium cohnii* fermentation using many different methods.

Species	X (g L ⁻¹)	L (g L ⁻¹)	D (g L ⁻¹)	T (h)	Culture	Medium	Reference
<i>S. limacinum</i>	58	37	12.7 ^a	350	Retention	Aqua-waste effluent	This study
<i>S. limacinum</i> OUC88	27		7.8	120	Batch	Defined	Song et al. (2007)
<i>S. limacinum</i>	22–24		4.8–4.9	120	Batch	B-Crude Glycerol	Chi et al. (2007)
<i>S. mangrovei</i>	7–14		0.75–2.8	52	Flask	Okara	Fan et al. (2001)
<i>S. mangrovei</i> SK-02	28	16	6	108	Batch	Coconut	Unagul et al. (2007)
<i>S. sp</i> G13/2S	63	16	5.5	50	Batch	Defined	Ganuza et al. (2008a,b)
<i>S. sp</i> G13/2S	6–8	2–3		70	Continuous	Defined	Ganuza and Izquierdo (2007)
<i>S. sp</i> S31	4–5	2	0.52–0.65	120	Flask	Defined	Wu et al. (2005)
<i>S. sp</i>	30	13	3.4	120	Batch	Shochu	Yamasaki et al. (2006)
<i>C. cohnii</i>	109	61	19	400	Fed-batch	Defined	Swaaf et al. (2003a,b)
<i>C. cohnii</i>	83	35	11.7	220	Fed-batch	Defined	Swaaf et al. (2003a,b)

X, biomass; L, Lipid; D, docosahexaenoic acid; T, time; Coconut, coconut water; B-crude glycerol, bio-diesel waste crude glycerol.

^a Long chain fatty acids (>C₂₂).

The utility of using this type of treatment system for RAS wastewater processing can now be discussed. A typical water exchange rate in a land-based marine RAS is about 5% per day of the recirculation water volume, below this level of exchange, there is potential for accumulation of materials that can significantly affect growth performance [Seginer et al., 2008; Good et al., 2009; Wolters et al., 2009]. At this exchange level most of wastewaters will be derived from the solids collection system and the subsequent related dewatering process and this stream can contain up to much of N and P in the effluents [Barak and Rijn, 2000]. The clarified waters from the dewatering process contain high concentrations of nutrients [Losordo et al., 2000; Summerfelt et al., 2004; Wolters et al., 2009] and this stream would be an appropriate point for *S. limacinum* nutrient removal treatment process, these flows are of relative low volumes that could easily pretreated (sterilization or pasteurization) and will contained enriched nutrients leached from sludge. Water treated in a cell retention system would then either be discharged or recycled, while the PUFA rich biomass of *S. limacinum* can be frequently harvested from treatment system and used as a source of valuable nutrients. The treatment capacity could be considerable and in our current work here where 420 g N, 190 g P and 10 g TA day⁻¹ m⁻³ (Table 5).

There remains one significant problem to this approach, namely the balancing of additional nutrients to those found in the waste. Good effluent treatment would also require that utilizable carbon must be at a minimum so that there is low BOD in the treated effluent water, i.e. glycerol and yeast extract additions would have balanced against the nutrients in the effluent water so that balances utilization of the materials occurs. For example, it could be envisaged that the system was in carbon access leading to substantial amounts of glycerol leaving the system in the effluent. This could be subsequently treated within wetland lagoons or avoided by carefully controlling the addition of glycerol and yeast extract. A calculation of the nutrient required can be made if the yield of biomass on glycerol is known. For example, a biomass yield on glycerol on a similar media (seawater 10 g L⁻¹ yeast extract) has been shown to be 0.5 g cell DW per g glycerol [Yokochi et al., 1998]. In our studies about we have shown that the yield was about 0.11 in the presence of 1 g L⁻¹ yeast extract supplement wastewater (Figure 3). In our studies although we have not measured the carbon content of the effluent an estimate can be made on the basis of cell yield in continuous culture. In Table 2 we report that 4.3 g biomass were produced on 10 g/L glycerol. If glycerol was the main carbon source used in this experiment, and using published yield value of 0.5 g/g, then 8.6 g of glycerol would be required. As there is no growth in the waste without carbohydrate addition and the fact that there is some inhibition compared to clean sea water (Fig. 2) it would seem reasonable to expect the glycerol levels remaining to be <1 g/L. If the yield derived from Fig. 3 was used then the system also most certainly limited by glycerol.

In conclusion this study has shown that *S. limacinum* could be produced as a high value biomass product containing long chain fatty acid-rich PUFA during the treatment of low volumes concentrated wastewaters processes such those derived from dewatering of sludge. The volumetric treatment and production capacity of the integrated system in retention culture was enhanced 13.5 times that of equivalent batch cultures. The hygienic aspects of this process have to be investigated further if this product were to be used an aquaculture feed.

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