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Key Selections for Microalgae, the Indispensable Live Feed in Bivalve Hatchery: A Brief Review

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ABSTRACT

In the current study, bivalve primarily consumed microalgae were addressed in all their developmental stages. Microalgae, an absolute nutrient source of essential phytonutrients and biologically active compounds, are vital for bivalve growth, health and reproduction. However, in natural habitat, its feeding preferences are not limited to only microalgae, but also include bacterioplankton, microzooplankton, detritus, protist as well as non-nutritive and dissolved organic materials. In contrast, bivalve feeding under controlled hatchery conditions relies on selective microalgae species for broodstock conditioning, larval and post-larval rearing. These activities mainly targeted the high production of seed, also known as spat which depends on the quality and quantity of the microalgae. Thus, thorough preparation and selection of microalgae are critical for the success of hatchery operations. This paper provides a brief overview of the current literature and understanding of microalgae selection criteria as live feed in bivalve hatchery.

INTRODUCTION

Microalgae, many referred to as phytoplankton, are a group of fast-growing microscopic unicellular or simple multi-cellular plant-like organisms **(Sostaric *et al.*, 2009; Creswell, 2010)**. It is a member of the enormous algae group, and can be found colonizing a wide range of habitats; freshwater, marine, and brackish water, such as oceans, rivers, lakes, and some exceptional microalgae can propagate in wastewater (**Khan *et al.*, 2018; Ilavarasi *et al.*, 2011; Scholz & Liebezeit, 2012**). Taxonomically, there are approximately 80,000 species of microalgae (**Parvin *et al.*, 2007**) classified into distinct groups comprise of diatoms (bacillariophyta), dinoflagellates (dinophyta), green and yellow-brown flagellates (chlorophyta; prasinophyta; prymnesiophyta, cryptophyta, chrysophyta, and rhaphidiophyta) and blue-green algae (cyanophyta) (**El Gamal, 2010**). Yet, miniature in size plays a momentous role in the self-sustaining and functional ecosystem on earth; microalgae act as the primary producer in the interconnected food

chains, both marine and freshwater environment. It is the basic unit of classical multitrophic food web, hence, allows the linear pathways of energy-flow and nutritional transfer between (in) water and (on) land organism, commonly, human, as the end consumer (**Napiorkowska-Krzebietke, 2017**).

Similar to conventional plants, microalgae are photoautotrophs. They convert inorganic compounds of water and carbon dioxide into organic biomass with the aid of light energy (**McCosh, 1984; Ilavarasi *et al.*, 2011; Masojidek *et al.*, 2013; Randrianarison & Ashraf, 2017**), but with higher photosynthetic efficiency, growth rate and biomass production (**Randrianarison & Ashraf, 2017**). While propagating in number, microalgae manufactured highly pharmaceutical-valued compounds as by-products (Fig. 1). These ingredients are either prized as whole-cell or extracted to produce supplements, pigments or medicinal purposes (**Creswell, 2010; Khan *et al.*, 2018**). Recent biotechnology advancement in biorefinery processing has been achieved to renounce recognition for microalgae as an interesting tool that can be manipulated to catalyst the biofuel production for cheaper and renewable energy (**Sostaric *et al.*, 2009; Creswell, 2010; Khan *et al.*, 2018**). Furthermore, likewise in its natural habitat, microalgae serve as an indispensable live feed in mariculture, especially in bivalve hatchery.

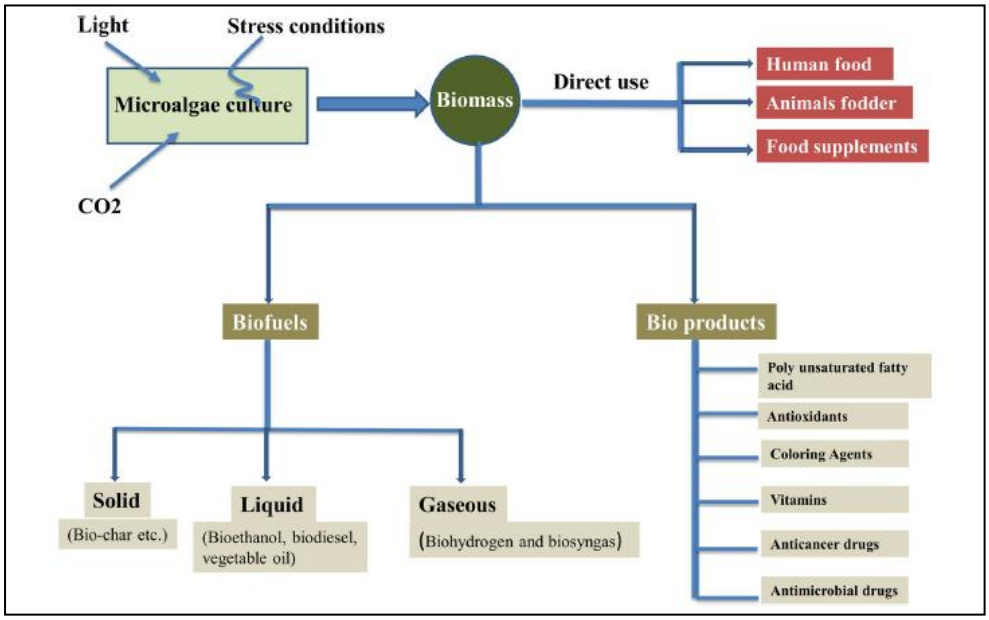

Fig. 1. Microalgae photosynthesize to produce biomass and by-products useful for feed, pharmaceutical, biofuel and medicinal purposes (**Khan *et al.*, 2018**)

The earliest cultivation of microalgae, as live feed in bivalve hatchery, has been recorded since the 1940s (**Bruce *et al.*, 1940**). Compared to their role as diets in the early development for various species of abalone and crustacean as well as a few species of fish, microalgae are inevitably consumed by bivalve in all its life cycle developmental

stages (**Brown, 2002; Rico-Villa *et al.*, 2006; Creswell, 2010**) (Fig. 2), specifically, the larval, post-larval (spat), juvenile and adult stages. Microalgae are an absolute nutrient source of essential phytonutrients and biologically active compounds, such as fatty acids, amino acids, sterols, organic minerals, enzymes, carotenoids, chlorophyll, trace elements and vitamins (**Napiorkowska-Krzebietke, 2017**). Hence, microalgae are vital to support bivalve growth, health and reproductive function.

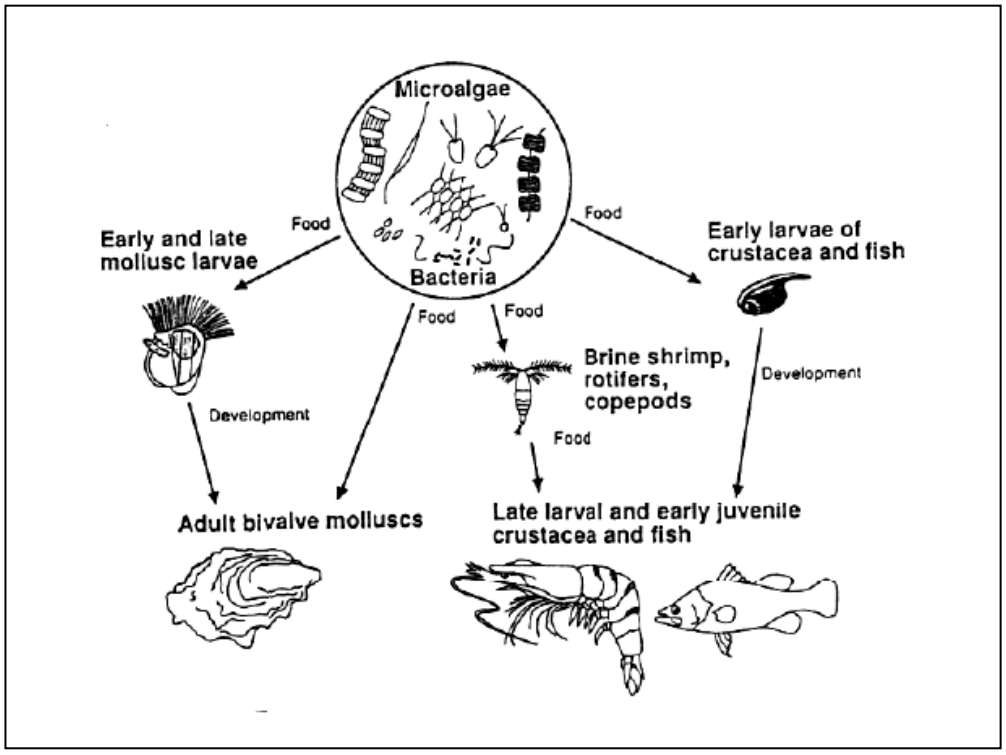

Fig. 2. The centralized role of microalgae in mariculture (**Brown *et al.*, 1989**)

Generally, bivalves; clams, oysters, mussels and scallops; are suspension feeders that use labial palps and gills (**Gosling, 2003; Beninger & Decottignies, 2005**) to discriminate between the fine particles in seston. Initially, bivalves are regarded as herbivorous animals, yet **Robinson *et al.* (2002)** investigated the gut content of blue mussels (*Mytilus edulis*) after the inclusion of zooplankton into their diets and confirmed the ingestion of copepods, tintinnids, and even bivalve larvae. To sum it up, bivalve wide feeding preference are not exclusively on microalgae but rather includes bacterioplankton, micro-zooplankton, detritus, protist, non-nutritive materials such as silt as well as dissolved organic material (DOM), for example, amino acids and sugars (**Robinson *et al.*, 2002; Gosling, 2003; Hwang *et al.*, 2004**). Addingly, they range up to 110 µm in size (**Newell *et al.*, 1989**). Large particles are rejected in the form of pseudofeces.

Conversely, the co-existence of various suspended particles in the water column is annulled under hatchery conditions as the water quality parameters, both physiological and nutrient were both controlled and sterilized, respectively. Bivalve hatchery operation traditionally practiced the collection of reproductively matured adults, referred to as broodstock, from the wild. The broodstock was induced to spawn for the gametes to be artificially fertilized and hatched as larvae. While some hatcheries are focusing on seed production, that is the rearing of the fertilized egg to reach eyed larvae (300-500 um) or small post-larvae (spat) (1-2 mm) for remote setting. Bivalve nursery rear juveniles beginning from 2 mm to the planting size; about 4 to 15 mm for clams; 5 to 30 mm for oysters and scallops; 15 to 20 cm for giant clam (**Coutteau & Sorgeloos, 1992**). Additionally, bivalve broodstock may be conditioned before spawning induction for a better quality of eggs and subsequently increase the larvae survival (**Gonzalez-Araya *et al.*, 2012**) preceding embryogenic development. More than that, protandric bivalve, such as oyster, *Ostrea edulis*, is greatly influenced by the types of microalgae species fed during conditioning (**Gonzalez-Araya *et al.*, 2013**). Thus, proper quality and quantity of microalgae as live feed need to be carefully chosen, otherwise, the hatchery operation would fail.

Due to the cost involved, the feeding strategy for hatchery-reared bivalves emphasizes the use of selected artificially cultured microalgae, to the least species possible, either single (monospecies) or a combination of two (bispecies) and three (trispecies) microalgae species. Thus, the selection of suitable microalgae for bivalve consumption is one of the most critical steps in setting up a hatchery. In the coastal and intertidal zone, natural microalgae selection for bivalve feeding is largely influenced by environmental parameters such as depth, tidal current, and filtration rate (**Bayne & Hawkins, 1988**) while bivalve reared in hatchery condition are more affected by the characteristics or physiology of the microalgae. Therefore, this paper intended to briefly review the factors that potentially mediate the microalgae selection as live feed in bivalve hatchery, particularly the evaluation and understanding of (1) nutritional value, (2) culture condition, and (3) cell size of the microalgae.

MICROALGAE SELECTION IN BIVALVE HATCHERY

Despite the enormous microalgae diversity (**Parvin *et al.*, 2007**) and substantial reviews on bivalve feeding mechanisms (**Gosling, 2003; Arapov *et al.*, 2010**), the knowledge on its feeding preference remains obscure (**Kasim and Mukai, 2009**). Yet, its microalgae selection as live feed in the hatchery, conventionally, is derived from the combination that is routinely used, regardless of bivalve species (**Robert *et al.*, 1994**). Other available options are to rely on reports of the wild bivalve dietary information available through the straightforward and robust method of gut content analysis. However, most of the time, the data obtained were vaguely biased due to the differences in feed digestibility (**Kasim & Mukai, 2009**). Henceforth, Maloy and colleagues have

attempted and successfully improvised the data collection by using a DNA-based approach by targeting 18S rRNA gene to screen all possible diets of bivalve larvae from marine waters (**Maloy *et al.*, 2013**). Such information is useful to clarify the naturally selected microalgae species, hinting at the suitable microalgae diets anticipated in the hatchery (**Kasim & Mukai, 2009**).

Bivalve feeding in hatchery targets the best microalgae species that support the growth and the survival of broodstock during conditioning, larvae rearing and spat growth (**Sanchez-Lazo & Martinez-Pita, 2014**) as well as broodstock post-spawn recovery. For cost-effective and ease of work, the type of feed is reduced to the least species possible, either single (monospecies) or a combination of two (bispecies) and three (trispecies) microalgae. Deciding on satisfactory live feed can be difficult and trampling. Gonzalez-Araya and his colleagues stated that the explicit traits of the best microalgae species for hatchery purposes are highly ingested, digested, assimilated, and efficiently allocated to the reproductive compartment (**Gonzalez-Araya *et al.*, 2012**). For this reason, there are 50-60 commercially available microalgae species (**Parvin *et al.*, 2007**), predominantly diatoms and dinoflagellates (**Sanchez-Lazo & Martinez-Pita, 2014**). In this context, only fewer than 10 species are extensively being used (**Coutteau & Sorgeloos, 1992**). More than that, the search for more 'wonder microalgae' to be used as a live feed candidate that supports better growth is a constant quest to answer. While the trial-and-error method is utilized in winning this chase, three predetermined factors that possibly influence microalgae selection as live feed in the hatchery are (1) nutritional value, (2) culture condition, and (3) cell size of the microalgae (**Creswell, 2010**).

1. Nutritional value

Foremost, microalgae nutritional value is the all-inclusive list of essential nutrients ratio, particularly, proteins, lipids, carbohydrates, minerals, and vitamins, that are required for an organism to attain the optimal body functions and metabolic processes (**Lofgren, 2013**). It allows a quick quality check of microalgae that vary by different species and culture conditions (**Napiorkowska-Krzebietke, 2017**). It is measured by cell size, digestibility, toxic secretion, and proximate content (**Brown, 2002**). The three most important nutrient fractions in nutritional value are protein, lipid, and carbohydrates, constituting about 12-35 %, 7.2-23 %, and 4.6-27 % of microalgae dry weight, respectively (**FAO, 2020**). In accordance, these nutrients also represent the major nutrient reserve in bivalve storage tissues, namely, adductor muscle, digestive gland and mantle (**Saucedo *et al.*, 2002**). Bivalve needs to actively mobilize energy from these storage cells to accommodate energy-requiring activities because, in many species, both growth and gonad development can occur simultaneously (**Mathieu & Lubet, 1992**).

Despite that, compared to lipid, there are mixed opinions on whether to support the role of proteins and carbohydrates as the determining factors for microalgae selection by bivalves. Proteins represent the largest nutrient fraction in microalgae, however, studies

stated that protein composition at the basic structural level, or amino acids, is very similar between microalgae-to-microalgae (**Brown, 1991**) and microalgae-to-bivalve (**Brown *et al.*, 1997**). Apart from being catabolized as a source of energy, protein also functions to supply nitrogen and essential amino acids for tissue biosynthesis (**Brown *et al.*, 1989**). Furthermore, most animals including fish and crustaceans require an external source of protein (**Li *et al.*, 2008**).It is worth noting that, bivalve in larvae form are shown to be able to perform *de novo* synthesis of essential amino acids; arginine, histidine, isoleucine, leucine, lysine, and valine (**Manahan, 1990**) for its usage, despite no sufficient data to support this proses.

Additionally, carbohydrate composition showed no major differences between species and classes of microalgae (**Brown, 1991**). Metabolically important, sugars were considered to balance between the food availability and energetic demand for reproduction and growth in bivalves (**Gonzalez-Araya *et al.*, 2011**). Sugar is catabolized as energy for gametogenesis (**Mathieu & Lubet, 1992; Gonzalez-Araya *et al.*, 2011**). It also acts as a precursor for lipid *de novo* synthesis during vitellogenesis (**Gabbott, 1983**). A similar finding was also reported in the study of **Galap *et al.* (1997)**, where changes were detected in muscular tissues (adductor muscle, foot, tunic coat) of cockle, *Glymcymeris glycymeris*, during vitellogenesis. The previous authors described that the major nutrient composition of these tissues, within 2-3 months before spawning indicates a high concentration of glycogen but recalculation about 1 month and just prior to designated spawning showed an increase in protein and lipid, respectively (**Galap *et al.*, 1997**). Despite that, **Chu and Greaves (1991)** proved that lipogenesis in bivalves is unable to synthesize one subgroup of lipid, that is the polyunsaturated fatty acids (PUFAs).

Generally, microalgae crude lipid composition consists of cholesterol, triglycerides, phospholipids and fatty acids (**Napiorkowska-Krzebietke, 2017**). Furthermore, fatty acids encompass a mixture of three subgroups, saturated fatty acid (SAFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). Each contains derivatives that are important to bivalves. According to **Brett *et al.* (2009)**, fatty acids content varies by microalgae species and origin; PUFAs were the highest component of fatty acids in many species of freshwater and marine chlorophytes and cryptophytes, as well as marine diatoms, while MUFAs and SAFAs contents recorded the highest in freshwater diatoms and cyanobacteria, respectively.

PUFAs are widely recognized as the determinant factor of the prime nutritive quality of microalgae (**Delaporte *et al.*, 2003**). It forms the precursor for the structural component of the cell membrane, hormones, pheromones, vitamins and pigments (**Hafezieh *et al.*, 2009**). The two types of PUFAs present in bivalve eggs, namely, phospholipids and neutral lipids, are obtained from two distinct sources, specifically during broodstock conditioning and reserved energy available onset of gametogenesis,

and during late-development of an oocyte, respectively (**Utting & Millican, 1997**). Two most well-known examples of PUFAs are eicosapentaenoic acid (EPA) (20: 5n-3) and docosahexaenoic acid (DHA) (22: 6n-3) (**Rico-Villa *et al.*, 2006**). Both are utilized as energy during embryogenesis until larvae can feed on exogenous food and used as a structural compound, respectively (**Hendriks *et al.*, 2003; Narvaez *et al.*, 2008**). Moreover, arachidonic acid (AA) (20: 4n-6) involves in regulating EPA synthesis as well as bivalve response to stress (**Pernet *et al.*, 2005**). Unsurprisingly, PUFAs content varies according to microalgae taxonomic groups (Fig. 3) (**Brown, 2002**).

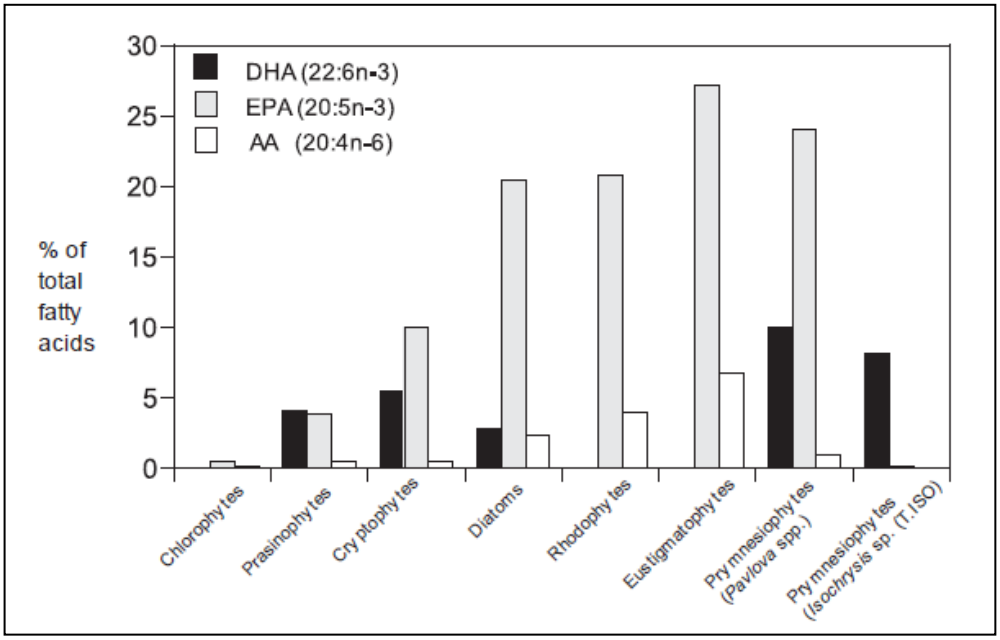

Fig. 3. Average percentage composition of three PUFAs derivatives, EPA (eicosapentaenoic acid), AA (arachidonic acid), and DHA (docosahexaenoic acid) from 46 species of microalgae according to its taxonomic group (**Brown, 2002**)

Apart from that, SAFAs and MUFAs are also houses for several important derivatives, namely, myristic acid (14:0), palmitoleic acid (16:1n-7), and oleic acid (18:1n-9), respectively. In their study, **Martinez-Pita *et al.* (2014)** proposed that mussels *Mytilus galloprovincialis* appeared to mobilize these derivatives from storage tissues into the reproductive cells during broodstock conditioning as reserved energy. This finding concurs with that of **Thompson *et al.* (1993)** who stated that, SAFAs are more ready to release the energy than their unsaturated equivalent counterparts during embryonic and larval development of Pacific oyster, *Crassostrea gigas*.

Consequently, the use of nutritional value information to select suitable microalgae as live feed in bivalve hatchery allows the customization of species-specific feeding type (**Brown, 1991**) as well as developmental-stage-specific feeding type to bivalve. For example, protein dietary requirement for spat rearing of clam, *Ruditapes decussatus* is 13

%; that is 7 % lower than oyster, *Crassostrea virginica* (**Flaak & Epifanio, 1978; Albentosa *et al.*, 1996**). Bu,t it is generally agreed that a combination of at least two, commonly, one diatom and one flagellate microalgae are necessary to achieve a well-balanced nutritional value live feed for bivalve culture (**Brown *et aI.*, 1997; Sanchez-Lazo & Martinez-Pita, 2014**).

2. Culture Condition

Following the nutritional value, well-defined biotic and abiotic elements to prepare a conducive environment for mass-production, or simplified as culture conditions, is the second most influential factor for live feed selection in bivalve hatchery. Worth mentioning, due to the cost-intensive, labor-intensive with specialized facility requirements (**Aji, 2011**) alongside execution time issues, the production of microalgae, so far, remains the main bottleneck for the bivalve hatchery set-up (**Coutteau and Sorgeloos, 1992**). The authors also deduced that microalgae operational cost devours up to 30-50 % of the total bivalve production cost. To provide comprehensive data,, this paper will divide all the parameters involved in the bivalve production into three parts: the upstream, midstream, and downstream processes (Fig. 4).

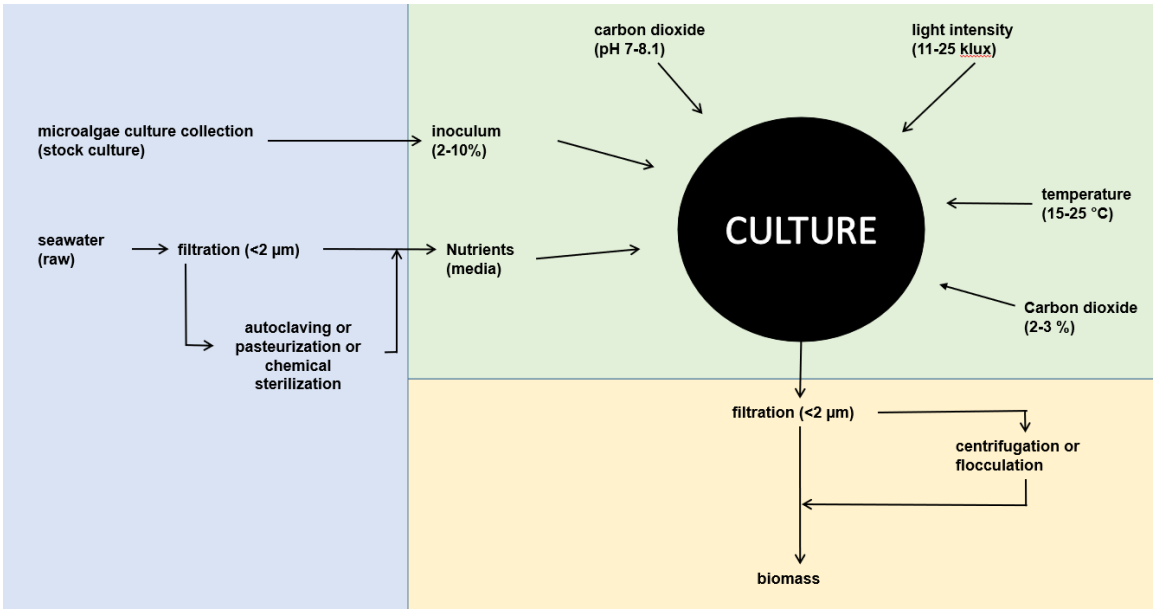

Fig. 4. Input requirements and environmental criteria for microalgae cultures; blue-upstream process, green-midstream process, yellow-downstream process (modified from **Creswell, 2010**)

The upstream process accounts for microalgae acquisition and culture water sterilization method. Microalgae axenic culture can be either locally isolated or commercially purchased from an established culture center. Isolation of endemic microalgae species from concentrated filtrates is performed using plankton net with a

mesh size of 10-120 µm (**Parvin *et al.*, 2007**). Pure culture deriving from single-celled microalgae species, termed as unialgal culture, is then established via several isolation techniques; micropipette washing technique (micromanipulation), centrifuge washing, and streak plating technique, serial dilution, and phototactic manipulation (**Parvin *et al.*, 2007; Perumal *et al.*, 2012**). For feeding purposes, the species of interest may not be necessarily representative of the *in situ* microalgae diversity population abundance, rather rapidly proliferate in an artificial and controlled environment. As an alternative, there are 50-60 commercially available microalgae species, although, only fewer than 10 species have been extensively cultured in bivalve hatchery (**Coutteau & Sorgeloos, 1992**) for the past 40 years. Those identified specieswere as such: *Isochrysis galbana*, *Isochrysis* sp. (T.ISO), *Pavlova lutheri*, *Tetraselmis suecica*, *Pseudoisochrysis paradoxa*, *Chaetoceros calcitrans* and *Skeletonema costatum* (**Persoone & Claus, 1980**). Regardlessly, the primary culture (stock culture) needs regular maintenance every two weeks while starter culture (inoculum) is sub-cultured from stock culture ahead of the initiation of mass production. To prevent bacteria and fungi contamination throughout the cultivation process, impurities removal from culture water is a prerequisite step. The sterilization method is dependent on the volume; maintaining a culture of less or equal to 2 liters is preferably done by filtration and followed by autoclaving method (**Creswell, 2010**). Meanwhile, cultivation of a large volume of microalgae requires a more practical and easy method such as using filtration followed by either chemical sterilization (e.g., commercial bleach; ratio 10 % of the volume) or pasteurization method (**Creswell, 2010**).

Next, the midstream process comprises the overall abiotic components to cultivate the microalgae including the selection of media and the determination of culture profile, scaling up method as well as facility design. The art of culturing for a particular species at this stage varies between hatcheries. Culture media are a formulation of essential macro- and micro-nutrients (trace elements and vitamins) required for microalgae to propagate. One can formulate a media by analyzing the water composition in microalgae's natural habitat, inspecting the nutrient requirement of specific microalgae, or improvising the existing version recipe (**Watanabe, 2005)**. With regards to microalgae's original habitat, two types of culture media are freshwater-based (e.g., Bold Basal medium, Jaworski's medium, etc.) and marine-based (e.g., Guillard f/2 medium, Von Stosch medium, Walne medium, Conway medium, etc.). Nutrients from the media can enrich the seawater's chemical properties to facilitate microalgae reproduction by cell division. In conjunction, seawater physical properties also require adjustment into a certain range of values to prevent environmental fluctuation and subsequently stabilize the cultivation process, creating culture profiles that are used repeatedly within the up-scaling production. Common culture profile used to mass-produced microalgae for feeding purposes comprises of light density (11 840 – 25 000 lux) for either continuous or alternate cycle between light: dark (16:8 h or 12:12 h, temperature (15-25 ºC), salinity (28-34 ppt) and with aerated with 2-3 % CO2 /air to maintain the pH (7-8.1) (**Brown *et al.*, 1991;**

Bertnsson *et al.*, 1997; Robert *et al.*, 2004; Creswell, 2010; Gonzalez-Araya *et al.*, 2011; Lananan *et al.*, 2013). Additional aeration and agitation may be necessary for diatoms as they tend to settle at the bottom.

After the initiation of microalgae culture, mass-production is achieved by scaling up the process in stages and is done in three types of culture system: batch, semi-continuous and continuous culture. Most bivalve hatcheries applied batch culture for *in situ* production of microalgae, with very little interest in the semi-continuous culture system (**Couteau & Sorgeloos, 1992**). In batch up-scaling culture, concentrated inoculum, either in exponential or the beginning of the stationary phase, were progressively transferred from one vessel into another, by which the total volume of previous is about 2-10 % (v/v) of the next vessel, and the biomass produced in final desirable culture vessel were harvested completely (**Creswell, 2010; Perumal *et al.*, 2012**). In the same review, Perumal and associates also described that, even though batch culture is a straightforward and flexible system fit to culture diverse species of microalgae and allow quick response to problems that may arise during the process, it produces an unpredictable quality of the microalgae biomass and the entire operation require a substantial amount of time and work to initiate as well as prone to contamination, especially during inoculation step (**Perumal *et al.*, 2012**).

Following that, scaling up production takes forms in two types of facility design, viz, suspension culture (open pond, enclosed reactor, and hybrid system) and immobilized cultures (biofilm and matrix-immobilized system) (**Randrianarison & Ashraf, 2017**). Enclosed reactor with applied batch culture system is frequent for small scale production or hatchery for academic purpose where microalgae were cultured in 20 L polyethylene terephthalate and 200 L fiberglass tank. However, a new type of enclosed reactor developed, Advanced Algal Production systems (AAPs) or photobioreactor, allows a continuous or semi-continuous culture of higher microalgae densities in small space that is less prone to contamination (**Creswell, 2010**). Yet, most commercial microalgae production widely applies the open pond system such as lagoons, lakes, ponds and raceways (**Randrianarison & Ashraf, 2017**).

In the downstream process lie the final elements to culture condition specifically the harvesting method. Harvesting, or collection of microalgae biomass, is performed when it reaches the late-exponential or early-stationary phase which is commonly, depending on species, achieved within 4-7 days (**Creswell, 2010**) and 8-7 days respectively. While some hatcheries directly feed bivalves with these cultures, the most convenient method is first to concentrate the microalgae into filtrates at 10^7-10^9 cells per mL and keep them in the 4°C until further use. The former may avoid the adverse effect of the microalgae quality due to processing and storage (**Hendriks *et al.*, 2003**), but may unintentionally introduce harmful ectocrine by-products and bacteria contaminant into the rearing tank. For harvesting and concentrating microalgae cultures, small-scale production often

applied filtration method while large and commercialized production make use of either centrifugation or flocculation method to speed up the recovery process (**Creswell, 2010**).

The selection of microalgae based on their culture condition is important as bivalve development is closely related to the quantity and quality of microalgae used as feed (**Rico-villa *et al.*, 2006**). Optimal culture condition is chosen based on microalgae growth performance. The three most observed growth indicators are the specific growth rate (relative ecological success of microalgae to adapt in the designated experimental environment) (**Levasseur *et al.*, 1993**), mother-daughter intercept (relative ratio of reproduction rate and mortality rate of microalgae), and the maximum cell density achieved during culture (**Lananan *et al.*, 2013**). Optimization for fast growth, high cell density, and improved nutritional value is possible through manipulation of chemical and physical properties of media. Often media, for instance, are designed to support general culture works, however, a study of **Lananan *et al.* (2013)** further deduced that inevitably, microalgae propagation is shown to thrive in selected media. Some not only show better growth performance in terms of higher cell densities and lipid production (**Sostaric *et al.*, 2009; Arkronrat *et al.*, 2016**) but also efficiently propagate and shift its population dynamics to a shorter period of culture (**Sostaric *et al.*, 2009**). Apart from that, changes in light intensity were found to improve the production SAFAs in microalgae (**Thompson *et al.*, 1993**). These are especially beneficial when rearing bivalve in the different developmental stages because feed needs to be allocated efficiently between the rearing of larvae and post-larvae as well as broodstock conditioning in the percentages of 10, 60, and 30 from total microalgae production, respectively. For comparison, estimated and sole development of an oyster egg into marketable size will consume 1.28×10^{12} cells of *Thallassiosira pseudonana*, which equivalent to 250 liters of dense microalgae culture (**Coutteau & Sorgeloos, 1992**).

3. Size

Finally, microalgae size is also an important variable to be considered when selecting live feed for bivalve hatchery. Pioneering the theories for qualitative feed selection **(Hughes, 1975)**, bivalves, renowned filter feeders is fed exclusively on particles with size up to 110 µm (**Newell *et al.*, 1989**). The feeding progression that begins with particle collection and transportation and ends with particle rejection or ingestion is divided into two sections based on the feeding mode **(Ward & Shumway, 2004)**. For suspension feeders such as oyster, mussel, and clam, the primary particle selection occurs in gills followed by secondary selection occurring in palps before being proceeded to the stomach (**Ward & Shumway, 2004; Prasetiya, 2015)**. Meanwhile, for deposit feeders of several clam species, the primary selection only happens in the palps (**Ward & Shumway, 2004**). Regardless of this, **Rosa *et al.* (2018)** in her review reported that, there are two types of particle selections during bivalve feeding: an active selection that relies on an immediate physiological response by the ciliated or feeding organs to feeding

stimuli (e.g stress) and a passive selection that is cued by the physicochemical interactions between the particles and the feeding organs (e.g size, shape, and cell concentration). Though it is suggested that the bivalve identifying these various particle sizes are in a mixture via the ligand-receptor relationship between glycan on microalgae cells surface and lectins presence on bivalve's gills and palp, still, the principles underlying the preferences and mechanism of feeding selection by size remain elusive (**Espinosa *et al.*, 2016; Rosa *et al.*, 2018**).

Nevertheless, evidence shows that the passive selection in bivalve feeding based on microalgae sizes is species-specific and life-stage-specific (**Jones & Gabbott, 1976**). For example, **Raby *et al.* (1996)** generalized the bivalve veliger's diet preferences as small-sized flagellates of <5 µm in size, but detailed observation by species shows that mussels have a wider size selection ranging from <5 µm to 25 µm followed by clam and scallops that only ingest microalgae size range from <5 µm to 15 µm. More than that, microalgae-size based feeding selection that also occurs within specific microalgae species but with different cell sizes as demonstrated by preferential rejection of adult oyster *Crassostrea gigas* towards larger cells (75 µm and 90 µm) of pennate diatom *Haslea ostrearia* but preferably ingesting smaller size of 50 µm of the same microalgae (**Prasetiya, 2015**). This selective behavior displayed by the bivalve towards small-sized microalgae is proposedly due to the gill architecture and cilia microstructure (**Rosa *et al.*, 2018**).

Compared to the early development of the stomach at the veliger larval stage, immediately as 20 hours post-fertilization (**Laxmilatha *et al.*, 2011**), the gills only appeared after 21 days post-fertilization, specifically, at the pediveliger larval stage, and completes the development at juvenile stage (**Laxmilatha *et al.*, 2011; Prasetiya, 2015**). There are two types of gills; homorhabdic gills in mussels and clams that are simpler in structure (flat and uniform series) but are less flexible than the heterohabdic gills found in oysters and scallops, as the latter structure are more complex (arranged in folds of plicae) and possesses expansible large filament opening (200um) (**Beninger *et al.*, 2004; Prasetiya *et al.*, 2015**) (Fig. 5). During feeding or respiration, suspended particles with water current first enter the infrabranchial chamber (mantle cavity), where large particles will be strained by latero-frontal cilia into frontal surface where it is wrapped in the mucus layer and transported along ventral ciliated particles groves towards labial palps before finally ejected as pseudofeces (**Gosling, 2003**). Meanwhile, finer particles pass through the inner side of the ciliated demibranch which forms the suprabranchial chamber (**Owen, 1974**) heading towards the stomach.

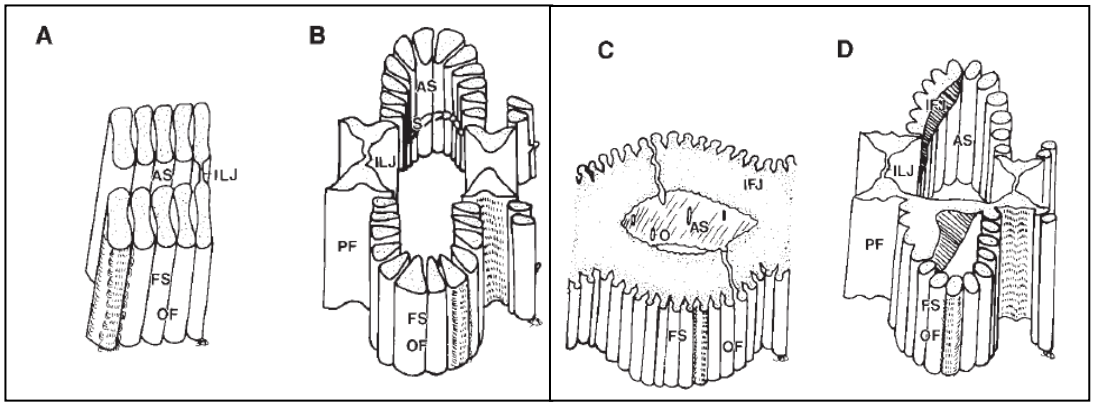

Fig. 5. Transverse section through the demibranchs of the principal gill types in feeding bivalves. **A.** Homorhabdic filibranch; ordinary filaments (OF) connected by interlamellar junctions (ILJ). B. Heterorhabdic filibranch; principal filaments (PF) and ordinary filaments, joined by interlamellar junctions and ciliated spurs (S). **C.** Homorhabdic eulamellibranch: ordinary filaments, joined by interfilament junctions (IFJ); O; ostia. **D.** Heterorhabdic pseodolamellibranch; principal filaments and ordinary filaments, joined by interlamellar junctions and interfilament junctions; AS abfrontal surface, FS frontal surface (Source: **Dufour & Beninger, 2001**).

Considering microalgae, sizes can prevent unnecessary organ damage to the bivalve during feeding, specifically to the ciliated microstructure in gills. When bivalves are given large-sized microalgae species, they are not only rejected,but cause severe loss of cilia. In this respect, **Cheung and Shin** (**2005**) showed that particles size between > 125 µm to < 250 µm led to significant loss of cilia caused by mechanical abrasion to the gill filaments of *Perna viridis*, whereas ingestion of particles < 63 µm in size are the least abrasive. With limited structural and functional ability, the bivalve at larval phase requires microalgae of < 10 µm with an optimal range of 2-5 µm to facilitate ingestion (**Robert & Trintignac, 1997**). Besides, the deliberate feeding also allows energy optimization by selective ingestion of microalgae with nourishing quality (**Rosa *et al.*, 2018**).

In terms of microalgae cultivation, sizes affect the efficiency of nutrient utilization and conversion into cell biomass. **Lananan *et al.*** (**2013**) based on microalgae specific growth rate, presumed that larger microalgae such as *Dunaliella* sp. (9.5 µm in diameter) and *Tetraselmis* sp. (8.5 µm) exhibited higher growth rate than small size microalgae such as *Chlorella* sp. (4.5 µm), *Pavlova* sp. (2.5 µm), *Isochrysis* sp. (2.5 µm) and *Chaetoceros* sp. (2.5 µm), but relatively with lower cell density, as larger cells required more nutrient for growth and reproduction. Subsequently, microalgae with a higher proliferation rate are likely less prone to be contaminated by bacteria thus ease the handling process. Besides, the same authors revealed that different microalgae sizes sustained different lengths of the period at the senescent phase as *Dunaliella* sp. was only

able to maintain stationary growth for a maximum of 2-3 days compared to 7-10 days in smaller microalgae size (**Lananan *et al.*, 2013**). Yet, prolonged exponential or stationary phase affects microalgae nutritional value.

CONCLUSION

Bivalves are an appealing source of protein used to cater the needs of ever-expanding human population. Thus, to reduce the dependency and prevent over-exploitation of natural stocks, hatchery production of spats is crucial for sustainable bivalve production. This can be achieved by having successful bivalve hatchery operation that is able to administrate suitable microalgae for feeding. The ideal characteristics for these microalgae must be easily culturable for bulk production whilst require only minimum handling, nutritionally balanced but rich in SAFAs and PUFAs as well as small (2-5 µm) in size to suit the feeding requirement during broodstock conditioning, and larval and post-larval rearing.

Table 1. The most frequently used microalgae in bivalve hatchery and studies related to its culture and production.

Microalgae used in bivalve hatchery	Size diameter (µm)	Nutritional content	Culture condition/ Remarks	Author (s)
Diatoms				
Chaetoceros sp. *Chaetoceros gracilis* *Chaetoceros calcitrans* *Chaetoceros neogracile*	2.5 - 4.4	Total SAFA, MUFA and PUFA are 22.04 - 49.5 %, 25.5 - 44.5 % and 11.19 - 47.08 %, respectively. Fatty acid composition in 100 % lipid consists of 5.7 - 21.44 % PA, 18.3 - 31.49 % POA, 0.5 - 1.5 % OA, 0.0 - 26.91 % EPA, 0.14 - 6.8 % ARA, 9.0 - 23.6 % MA, 0.0 - 2.1 % LA and 0.0 - 1.7 % DHA.	The strain can be isolated from environmental sample isolate. For feed, it is suitable for mass culture up to 300 L batch using f/2, Walne or Conway media. The culture profile used to culture is 21 - 25 °C, pH 7.6 - 8.1, 34 ppt, 11.8 - 12.5 Klux at 16:8 hours of light:dark cycles or continuous light with aeration as well as 2 or 25 % of volume per volume (v/v) inoculum. The culture was harvested at exponential or late-logarithmic phase with highest cell density achieved that varies by media, ranging from 4.0 x 10^6 cells/ mL to 1.2 x 10^7 cells/ mL, within 23 - 30 days of culture.	**Berntsson *et al.*, 1997; Caers *et al.*, 1998; Delaporte *et al.*, 2003; Rico-Villa *et al.*, 2006; Gonzalez-Araya *et al.*, 2011; Lananan *et al.*, 2013; Sanchez-Lazo and Martinez-Pita, 2014**
Thalassiosira spp. *Thalassiosira weissflogii* *Thalassiosira pseudonana*	-	Total MUFA and PUFA are 19.67 - 22.47 % and 16.82 - 47.08 %, respectively. Fatty acid composition in 100 % lipid consists of 13.6 - 24.05 % PA, 17.30 - 20.14 % POA, 0.0 - 0.99 % OA, 14.70 - 20.43 % EPA, 0.22 -	For feed, it can be cultured up to 300 L batch culture using Conway medium and harvested at 3-5 days in the late-logarithmic phase. But the culture condition was not specified.	**Gonzalez-Araya *et al.*, 2012**

		9.41 % ARA, 6.76 - 7.86 % MA 0.57 - 1.25 % LA and 3.60 - 4.64 % DHA.		
Skeletonema sp.	-	Total SAFA, MUFA and PUFA are 16.82 %, 15.48 % and 65.73 %, respectively. Fatty acid composition in 100 % lipid consists of 6.66 % PA, 13.44 % POA, 0.6 % OA, 21.88 % EPA, 0.43 % ARA, 9.64 % MA, 1.24 % LA, 4.4 % DHA.	For feed, it is suitable for mass culture up to 300 L batch culture using Conway medium cultured with culture profile of 11.8 Klux, 21 °C, pH 7.6-8.1, 34 ppt with 2 % (v/v) inoculum. The culture was harvested at 3-5 days in the late-logarithmic phase	**Gonzalez-Araya *et al.*, 2011**
Yellow-brown flagellates				
Isochrysis sp. (T-iso) *Isochrysis galbana* *Isochrysis affinis galbana*	2.5 - 4.4	Total SAFA, MUFA and PUFA are 29.48 - 51.2 %, 11.2 - 30.90 % and 28.22 - 48.37 %, respectively. Fatty acid composition in 100 % lipid consists of 11.9 - 22.0 % PA, 1.71 - 35.6 % POA, 3.8 - 22.01 % OA, 0.0 - 14.0 % EPA, 0.0 - 0.3 % ARA, 9.37 - 26.5 % MA, 3.1 - 11.62 % LA and 4.51 - 11.1 % DHA.	The strain can be commercially purchased or isolated from environmental sample. For feed, it is suitable for high volume culture up to 600 L, using batch or continuous methods as well as variety of media such as f/2, Walne or Conway media. The culture profiles used are 21 - 25 °C, pH 7.6 - 8.1, 34 ppt, 11.8 - 22 Klux at 16:8 hours of light: dark cycles or continuous light or artificial light with aeration and 2 or 25 % (v/v) inoculum. It was harvested at exponential or late-logarithmic phase with highest cell density achieved vary by media and range from 6.0 x 10^6 cells/mL to 2.3 x 10^7	**Berntsson *et al.*, 1997; Caers *et al.*, 1998; Delaporte *et al.*, 2003; Hendriks *et al.*, 2003; Rico-Villa *et al.*, 2006; Gonzalez-Araya *et al.*, 2011; Lananan *et al.*, 2013; Sanchez-Lazo**

			cells/mL, within 17 - 20 days of culture.	**and Martinez-Pita, 2014**
Tetraselmis sp. *Tetraselmis suecica*	8 - 8.5	In general, 21.7 - 55.2 % of protein, 14.5 - 20 % of carbohydrate, 9.4 - 20 % of lipid. Total SAFA, MUFA and PUFA are 26.1 - 65.2 %, 11.2 - 34.19 % and 7.4 - 53.2 %, respectively. Fatty acid composition in 100 % lipid consists of 23.67 - 30.82 % PA, 0.1 - 5.6 % POA, 0.6 - 21.51 % OA 2.3 - 7.2 % EPA, 0.37 - 1.2 % ARA 0.29 - 3.3 % MA, 2.0 - 14.6 % LA and 0.0 - 0.23 % DHA.	The strain can be commercially purchased or isolated from environmental sample. For feed, it is suitable for mass culture up to 600 L batch or continuous culture using f/2, Walne or Conway media. The culture profile used are Culture profile: 21-25 °C, pH 7.5-8.5, 28-34 ppt, 3-22 Klux using 24 hours continuous artificial photoperiod, combination of natural light and aeration as well as 2 or 25 % (v/v) inoculum. The culture was harvested at exponential, late-logarithmic or stationary phase with highest cell density achieved that varies by media, salinity and pH, ranging from 4 x 10^5 cells/ mL to 3.543 x 10^7 cells/ mL, within 25 - 30 days of culture.	**Berntsson *et al.*, 1997; Caers *et al.*, 1998; Delaporte *et al.*, 2003; Gonzalez-Araya *et al.*, 2011; Lananan *et al.*, 2013; Khatoon *et al.*, 2014; Sanchez-Lazo and Martinez-Pita, 2014; Arkronrat *et al.*, 2016**
Dunaliella sp. *Dunaliella tertiolecta*	9.5	Total SAFA and MUFA are 21.5 % and 14.4 %, respectively. Fatty acid composition in 100 % lipid consists of 19.2 % PA, 1.8 % POA, 7.1 % OA, 0.0 % EPA, 0.0 % ARA, 1.9 % MA, 13. 6 % LA and 0.0 % DHA.	The strain can be isolated from environmental sample. For feed, only small batch culture of 1-4 L using f/2, Walne or Conway media was produced. The culture profile is not specified except for temperature and photoperiod; 25 °C of continuous light; and 25 % (v/v) inoculum. The culture was harvested at exponential phase with highest cell density achieved and varied by media and ranged from 7.5 x 10^6 cells/mL to 8.2 x 10^6 cells/mL,	**Caers *et al.*, 1998; Lananan *et al.*, 2013**

			within 27 days of culture.	
Pavlova sp. *Pavlova lutheri*	2.5	Total MUFA and PUFA are 19.86 % and 49.41 %, respectively. Fatty acid composition in 100 % lipid consists of 19.45 % PA, 16.27 % POA, 1.31 % OA, 23.37 % EPA, 0.46 % ARA, 10.04 % MA, 2.42 % LA and 10.75 % DHA.	Culture source can be isolated from environmental sample. For feed, suitable for high volume batch culture up to 300 L using the f/2 or Conway media. The culture profile was not specified except temperature (25 °C) and 25 % (v/v) inoculum. It is harvested at late-logarithmic phase with highest cell density achieved is 2.2 x 10^7 cells/mL, within 20 - 30 days of culture.	**Gonzalez-Araya *et al.*, 2012; Lananan *et al.*, 2013;**
Chlorella sp. *Chlorella vulgaris*	4.5	Not specified	The strain can be obtained commercially strain or isolated from environmental sample. For feed, small batch culture up to 3 L using variety media such as f/2, Conway media, Jaworski media, modified Solvay process solution and mineral water Donat^{Mg,} was produced. The culture profiles used are 25°C, 7.4 - 8.9 Klux at 12:12 hours of light: dark cycles of artificial light and 25 % (v/v) inoculum. For harvesting, the highest cell density achieved varied by media and ranged from 7.8 x 10^6 cells/ mL to 2.2 x 10^7 cells/ mL, within 9 - 20 days of culture.	**Sostaric *et al.*, 2009; Lananan *et al.*, 2013;**
Rhodomonas salina	-	Total MUFA and PUFA are 8.35 % and 68.74 %, respectively.	For feed, it can be cultured in large volume of 300 L batch culture using 2x strength Conway medium, but the culture condition was not specified. The	**Gonzalez-Araya *et al.*, 2012**

		Fatty acid composition in 100 % lipid consists of 13.6 % PA, 0.74 % POA, 1.26 % OA, 9.51 % EPA, 2.41 % ARA, 7.26 % MA, 18.04 % LA, 8.18 % DHA.	culture was harvested at late logarithmic phase after 3-5 days culture.	
Blue-green algae				
Nannochloropsis sp.	2	In general, 22.5 - 45 % of protein, 10 - 35.7 % of carbohydrate, 11.1 - 30 % of lipid. Fatty acid composition in 100 % lipid consists of 5.05 % PA, 4.72 % POA, 3.79 % OA, 2.24 % EPA, 0.69 % ARA, 0.63 % MA, 0.36 % LA	The strain can be obtained commercially strain or isolated from environmental sample. For feed, small batch culture up to 29 L using f/2 or Conway media were produced. The culture profiles used are 20 - 25 °C, pH 7.5 - 8.5, 28 - 30 ppt, 1.6 - 8.5 Klux for 24 hours photoperiod using continuous light and natural light with aeration. The culture was harvested at stationary phase with highest cell density achieved ranging from17 x 10^5 cells/ mL to 4.877 x 10^7 cells/ mL, within 10 - 14.5 days of culture.	**Rebolloso-Fuentes *et al.*, 2001; Khatoon *et al.*, 2014; Arkronrat *et al.*, 2016**

PA - palmitic acid, POA - palmitoleic acid, OA - oleic acid, EPA - eicosapentaenoic acid, ARA - arachidonic acid, MA - myristic acid, LA - linoleic acid, DHA - docosahexaenoic acid, SAFA - saturated fatty acids, MUFA - monounsaturated fatty acids, PUFA - polyunsaturated fatty acids; µm - micrometer.

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