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PRODUCTION OF LIVE FOOD FROM PALM OIL MILL EFFLUENT (POME) FOR CULTURE OF MARBLE GOBY

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ABSTRACT

For the first time a phototrophic bacterium, Rhodovulum sulfidophilum *grown in palm oil mill effluent (POME-PB) was successfully used as food for rotifers* (Brachionus rotundiformis) *and* Artemia *nauplii which were then fed to the larvae of the marble goby,* Oxyeleotris marmorata. *The cultivation of POME-PB is cheap and easy where it can be easily produced in sealed, plastic ziplock bags exposed to light, giving a harvestable biomass of 2.58 ± 0.34 g litre^{-1} after 60 hr post-inoculation. Rotifers fed the biomass of settled bacteria (sPOME-PB) had comparable mean density (221 ± 9 rotifers ml^{-1}) as rotifers fed with microalgae* Nannochloropsis *sp. (212 ± 27 rotifers ml^{-1}). However, unsettled bacterial culture (uPOME-PB) fed to rotifers resulted in higher rotifer densities of up to 898 ± 489 rotifers ml^{-1}. By feeding the marble goby larvae with rotifers and* Artemia *nauplii cultured in uPOME-PB, a mean survival of 81.9 ± 3.0% was obtained for 30-day post-hatch fish larvae. This survival was much higher than fish larvae given rotifers and* Artemia *nauplii fed sPOME-PB (42.5 ± 9.0%; 71.4 ± 20.5%) and* Nannochloropsis *sp. (46.8 ± 2.9%). POME-PB thus has the potential to be an aquaculture feed for the future.*

Keywords: palm oil mill effluent, phototrophic bacteria, rotifers, marble goby culture.

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INTRODUCTION

Worldwide aquaculture produces 55.1 million tonnes of fish to supplement the ever increasing demand for fish food and protein, while wild catches continue to fall (FAO, 2010). The commercial production of artifical feed for cultured organisms however uses 71% and 90% of the world's fish meal and fish oil production, respectively (Tacon and Metian, 2008), and it is projected that aquaculture feed production will face serious shortages in the near future as fish meal production leveled off and fell since 1995 (James and Geoff, 2001). The search is now for alternative sources of proteins and omega 3-rich oils that could be mass cultured cheaply, harvested and used as feed for fish and other cultured organisms. Single-cell proteins (SCP) and oils (SCO) are the current viable sources that include unconventionally exploited microorganisms such as heterotrophic dinoflagellates, microheterotrophs and phototrophic bacteria (PB). Unlike microalgae, these organisms do not strictly need light and are more useful in bioremediating organic wastes, which if untreated, pollute the environment. One such waste is palm oil mill effluent (POME) generated in large volumes by Malaysia's largest agroindustry that contributes to 3.3% of its gross domestic product. It is estimated that for every tonne of crude palm oil produced, an equivalent 2.5 t of POME is generated (Department of Environment, 1999). Yet POME is nutrient and lipid rich (Phang, 1990).

A dual solution to overcome a potentially debilitating environmental problem caused by the palm oil industry and the woes of the aquaculture industry is to find that one 'magic bullet' that could mitigate the organic pollution while turning it into a nutriment for fish. That magic bullet could be a

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simple organism, PB, which have the potential to reduce the wastewater chemical oxygen demand by as much as 80% and giving a harvested biomass of as high as 60% protein content (Duangporn *et al*., 2005).

The application of PB as fish feed in the aquaculture industry is fairly recent because their discovery, versatility and benefits have only been recently appreciated. For instance, the protein content of PB cells (60%-70%) is superior to that of yeast (40%-60%) (Sasaki *et al*., 1998) and comparable to algae (50%-60%), soyabean meal and egg (Kobayashi and Kurata, 1978; Noparatnaraporn *et al*., 1987), commonly used feeds in aquaculture.

These commonly used feeds are seldom fed directly to the fish larvae they nourish, but via zooplankton which are the natural live food of fish larvae. Rotifers (*Brachionus* spp.) are important live food of fish larvae due to their small size and high digestibility (Theilacker and Kimball, 1984; Hoff and Snell, 1997). Nevertheless, the nutritional value of rotifers depends on their diet (Lubzens *et al*., 1995).

Current hatchery production of the valuable marble goby (*Oxyeleotris marmorata*) is very low due to poor survival rate of cultured larvae (Senoo *et al*., 2008) which appears to be the main bottleneck of large scale commercial production. This is believed to be due to suboptimal nutrition at the critical phase of its development. Hence, the objective of the present study is to investigate whether POME nutrients can be utilised to culture the phototropic bacterium, *Rhodovulum sulfidophilum* as a live feed for aquaculture, in particular rotifer and marble goby.

MATERIALS AND METHODS

Culture of Phototrophic Bacteria

The stock culture of PB, *Rhodovulum sulfidophilum* was obtained from Mycology and Plant Pathology Laboratory, University of Malaya. The master stock culture of the bacteria was maintained as stab culture on 112 medium (Gest and Favinger, 1983) solidified with 1.5% (w/v) agar. The stock culture was plated out and checked for purity prior to use.

Fresh POME from the separator at the MPOB Experimental Palm Oil Mill, Labu, Negeri Sembilan, Malaysia, was collected and kept in a cold room for a day prior to centrifuging the settled down liquid to obtain the supernatant. The supernatant was then filtered through 25 μm pore size Whatman paper to further discard solid particles prior to diluting it with distilled water in the ratio of 25% POME (v/v) and 75% distilled water. The diluted POME was autoclaved at 121°C and 15 psi for 15 min before use as a growth substrate for PB.

The PB inoculum was prepared in 112 medium. A loopful of four-day old pure bacterial culture was inoculated into each of several bottles containing autoclaved 112 medium. The bottled bacterial culture was incubated under anaerobic-light condition.

Rotifer (*Brachionus rotundiformis*) Culture

The stock of rotifer was obtained from the Department of Fisheries Malaysia. A rotifer density of 75 individuals ml^{-1} ($ind.ml^{-1}$) was cultured in 4-litre amber bottles with conical bottom. The corresponding volume of culture water used was 3 litres of 5 ppt salinity.

Marble Goby Culture

Larvae of marble goby were cultured for 30 days, a period considered to be the most critical phase of its life history. A pair of marble goby adults of F1 generation was used as broodstock. One day post-hatch (dph) fish larvae were reared in cylindrical tanks with conical bottom and working volume of 5 ppt salinity water. Rotifers were fed to fish larvae at the feeding rate of 10 rotifers ml^{-1} (Day 0 - Day 20) and five rotifers ml^{-1} (Day 21 - Day 30), while *Artemia* nauplii were given to fish larvae from Day 21 onwards at a density of 5 nauplii ml^{-1}. The *Artemia* nauplii were hatched from commercially produced cysts (Hong Da Artemia Cysts, China).

Experimental Designs

Production of* R. sulfidophilum *grown in POME (POME-PB). Ten percent of 1-litre PB grown in synthetic 112 medium was inoculated into diluted POME medium. The bacterial cultures were then incubated under anaerobic-light condition at 30 ± 2°C for 60 hr. The bacterial experiment tested the effect of type of culture vessel (1-litre Schott bottle or ziplock bag) on the production of POME-PB. The dry weight of the cell biomass of POME-PB (g $litre^{-1}$) was determined according to Sawada and Rogers (1977), while the fatty acid profile of freeze dried bacteria was determined using gas chromatography on a HP5890. Prior to fatty acid analysis, the bacteria were harvested after 60 hr post-inoculation and the biomass was washed several times with sterilised 0.9% (v/v) sodium chloride solution before freeze-drying. The fatty acid methyl esters were prepared based on the International Union of Pure and Applied Chemistry (IUPAC) method 2.301 (Anon, 1987). Two forms of bacterial feed cultured in POME were prepared for testing: uPOME-PB, which is the unsettled PB culture in residual POME and sPOME-PB which is the centrifuged biomass of PB. Only the settled form of bacteria cultured in 112 medium (s112-PB) was used as experimental feed.

Production of rotifers fed with POME-PB. Two rotifer production experiments were conducted to evaluate: 1) production density based on sPOME-PB, compared to commercial marine microalgae *Nannochloropsis* sp. (Nanno 3600™; Reed Mariculture, San Jose, CA, USA) a common rotifer diet, and 2) the comparative effects of sPOME-PB and uPOME-PB on rotifer production. The rotifers were given a total daily PB or microalgal ration of 100 ml (ca. 0.17 g dry weight) of the aqueous feed stock in the first experiment, and 200 ml of aqueous PB feed stock (ca. 0.34 g dry weight of organic matter) in the second experiment. The rotifer density was determined daily as number of ind. ml^{-1}, based on three sub-samples taken from each culture tank.

Survival and Growth of Larval Marble Goby

Larval rearing experiments were conducted to test: 1) the relative benefit of rotifers and *Artemia* nauplii (henceforth referred to as R-A) cultured from sPOME-PB to the survival and growth of marble goby larvae, compared to R-A fed with *Nannochloropsis* sp., and 2) the survival and growth of fish larvae fed R-A cultured from either uPOME-PB or sPOME-PB. The first experiment was conducted at larval fish stocking density of 10 larvae $litre^{-1}$, whereas the second experiment used a stocking density of 15 larvae $litre^{-1}$. The survival and growth (total length, TL) of fish larvae were determined at 10-day intervals over a culture period of 30 days.

Statistical Analysis

Student's *t* test was used to determine the effect of culture vessel (*i.e.* 1-litre Schott bottle *vs.* ziplock bag) on the production of POME-PB. The percentage of docosahaxaenoic acid (DHA) and eicosapentaenoic acid (EPA) in enriched bacteria (s112-PB *vs*. sPOME-PB) were respectively tested for significant difference using the *t*-test. Two-way ANOVA and post-hoc Tukey HSD test were used to determine the main effects of day of culture (Day 1, 2, …, 6) and feed type (sPOME-PB, *Nannochloropsis*) or (sPOME-PB, uPOME-PB) on rotifer peak production. The mean and standard deviation (SD) of survival (%) and TL (mm) of larval marble goby were calculated. The percent survival results were arcsine-transformed before parametric testing. The percent survival and TL of larval marble goby at the final day (Day 30) of experiment as affected by feed (R-A fed sPOME-PB against R-A fed *Nannochloropsis*) or (R-A fed sPOME-PB against R-A fed uPOME-PB) were tested using the *t*-test. Statistical analysis was done using Statistica, version 10.

RESULTS AND DISCUSSION

The Effect of Type of Culture Vessel on POME-PB Production

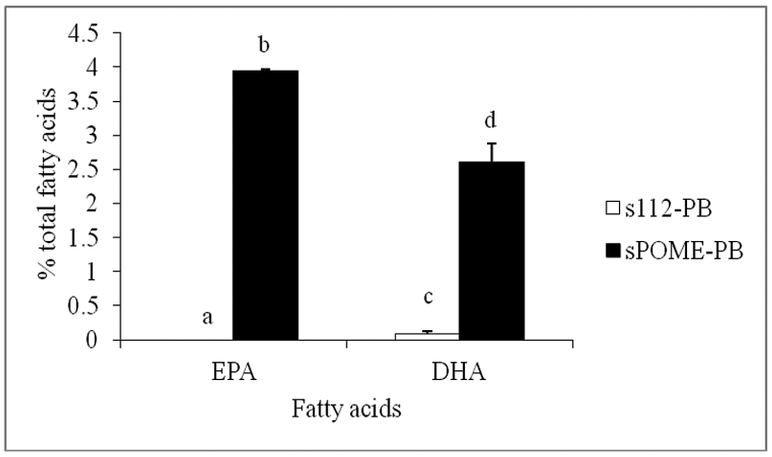
The type of culture vessel had an effect on bacterial yield. The POME-PB cultured in ziplock bags (2.58 ± 0.34 g $litre^{-1}$) had significantly higher (*t*-test, $P < 0.01$) mean dry biomass than bacteria grown in Schott bottles (1.18 ± 0.27 g $litre^{-1}$) after 60 hr post-inoculation. This effect is most likely due to the thick round wall, long diameter and lower surface to volume ratio of the Schott bottle (compared to the ziplock bag) which would have allowed less light transmission into the medium. According to Richmond (2004), the light that activates photosynthesis can only penetrate ca. 3 cm into a culture medium.

Fatty acid analysis demonstrates that *R. sulfidophilum* can extract and incorporate nutrients such as α-linolenic acid (0.9% of total fatty acids) from POME into their cells and utilise this precursor to synthesise EPA and DHA. This interpretation is based on the presence of significant (*t*-test, $P < 0.01$) amounts of EPA (3.94 ± 0.03%) and DHA (2.60 ± 0.28%) in sPOME-PB, as compared to s112-PB (0%; 0.04 ± 0.04%) (*Figure 1*). Both EPA and DHA are essential for larval fish survival and growth (Sargent *et al*., 1999).

The Effect of Feed Quality on Rotifer Production

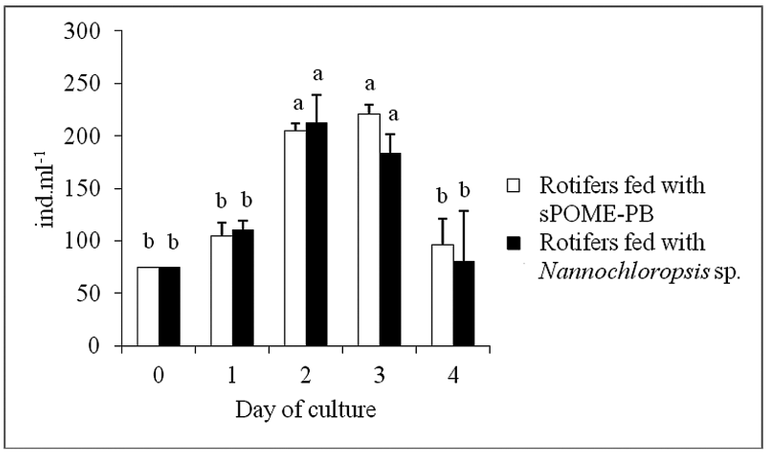
The rotifer peak production was only significantly affected (two-way ANOVA, $P < 0.01$) by day of culture [Day 2 (209 ind.ml^{-1}) = Day 3 (202 ind.ml^{-1}) > Day 1 (108 ind.ml^{-1}) = Day 4 (88 ind. ml^{-1}) = Day 0 (75 ind.ml^{-1})]. The peak densities of rotifers fed with sPOME-PB (221 ± 9 ind.ml^{-1}) and rotifers fed with *Nannochloropsis* (212 ± 27 ind.ml^{-1}) (*Figure 2*), were not significantly different (two-way ANOVA, $P > 0.05$). This shows that sPOME-PB is useful as a feed for culturing rotifers and can reduce present reliance on microalgae for rotifer culture.

The rotifer peak production was significantly affected (two-way ANOVA, $P < 0.01$) by the form of bacterial feed, day of culture and their interaction (day x feed). For instance, rotifers fed with uPOME-PB had the highest peak density of 898 ± 489 ind. ml^{-1} at Day 4 as compared to those fed with sPOME-PB (323 ± 51 ind.ml^{-1}) at Day 3 (*Figure 3*). However, rotifer cultures using uPOME-PB were more variable in density (*t*-test, $P < 0.01$) than those using sPOME-PB. This could be due to the higher mean levels of ammonia excreted by rotifers fed uPOME-PB (6.93 mg $litre^{-1}$) than rotifers fed sPOME-PB (4.05 mg $litre^{-1}$). The reason for this is not understood, although rotifers are known to excrete ammonia at levels depending on the type of feed given (Kiorboe



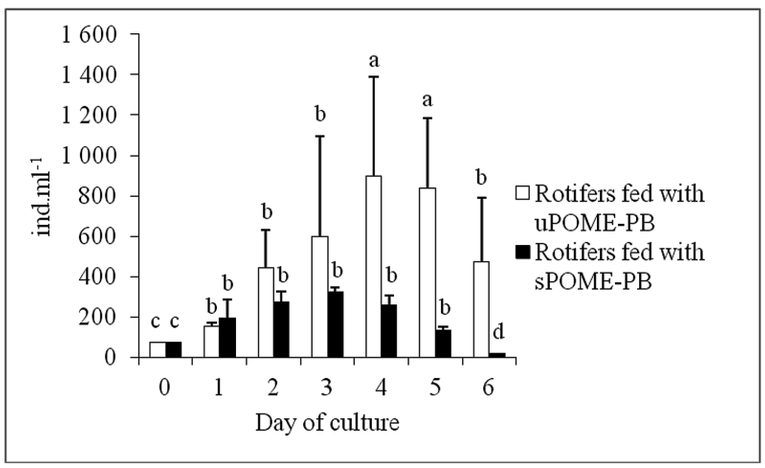
Note: [a,b,c,d]Indicate homogeneous groups in t-test (P > 0.05).

Figure 1. EPA and DHA composition in palm oil mill effluent (POME) grown bacteria (sPOME-PB), compared to bacteria grown in synthetic medium (s112-PB).



Note: [a,b]Indicate homogeneous groups in Tukey's test (P > 0.05).

Figure 2. Production density of rotifers (mean ± SD; n = 9) cultured for four days on biomass of settled bacteria (sPOME-PB), compared to Nannochloropsis *sp.*



Note: [a,b,c,d]Indicate homogeneous groups in Tukey's test (P > 0.05).

Figure 3. Production density of rotifers (mean ± SD; n = 9) cultured for six days on two different forms of bacterial diet (uPOME-PB and sPOME-PB).

et al., 1985). On the other hand, the residual POME or POME as present in uPOME-PB could not have raised the ammonia level in the rotifer culture since the mean ammonia level in tanks with rotifers fed POME alone (0.20 mg litre^{-1}) was the lowest.

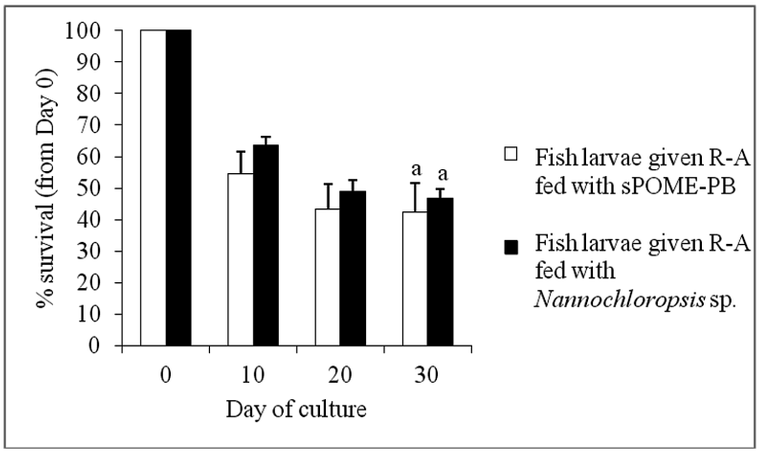
Rotifer production increased with higher food density, *i.e*. from 100 ml to 200 ml of sPOME-PB (*Figures 2* and *3*). Hence, the study shows that both PB and POME are non-toxic to rotifers and the residual POME (as in uPOME-PB) could have enhanced rotifer production. POME contains high amounts of lipids, protein, carbohydrate, minerals and nitrogenous compounds (Phang, 1990).

Survival and Growth of Larval Marble Goby

The survival (42.4 ± 9.1%) and growth (11.16 ± 1.05 mm TL) of 30 dph marble goby larvae given R-A fed with sPOME-PB were comparable (*t*-test, P > 0.05) to larvae given R-A fed with *Nannochloropsis* (46.8 ± 2.9%; 10.77 ± 0.55 mm TL) (*Figures 4* and *5*).

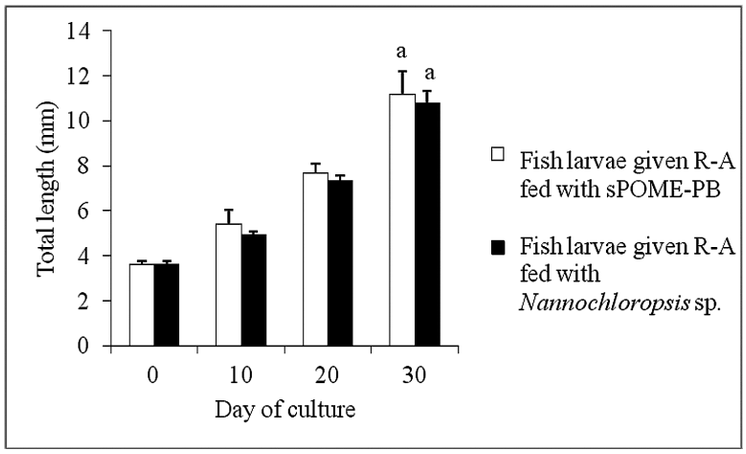
Larval survival was also not significantly affected (*t*-test, P > 0.05) by R-A fed with the two forms of enriched bacteria. At the end of the rearing period (Day 30), the mean survival for larval fish given R-A fed with uPOME-PB (81.9 ± 3.0%) was not significantly (*t*-test, P > 0.05) different from those given R-A fed with sPOME-PB (71.4 ± 20.5%) (*Figure 6*). However, the mean TL of 30 dph larvae given R-A fed with sPOME-PB (12.30 ± 0.52 mm) was significantly (*t*-test, P < 0.01) longer than those given R-A fed with uPOME-PB (11.07 ± 0.05 mm) (*Figure 7*).

The results obtained in this study using POME fed organisms to feed fish larvae are much better than those obtained in previous studies using R-A fed with *Nannochloropsis* sp. and cod oil juice; for instance, larval fish had only 10% survival and



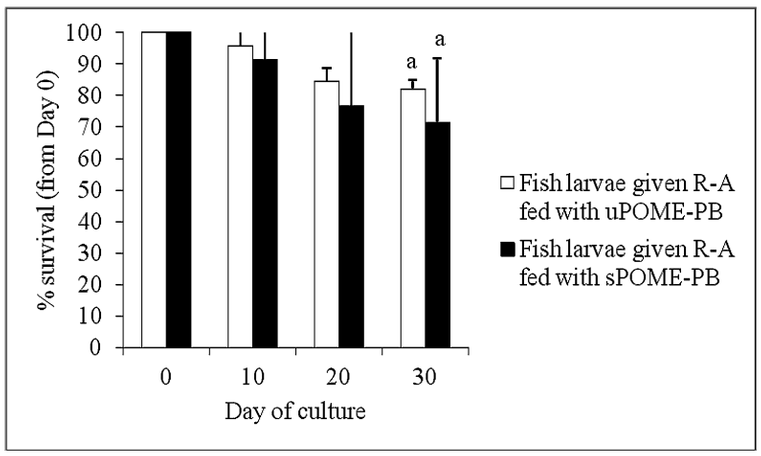
Note: [a]Indicates homogeneous group in t-test (P > 0.05).

Figure 4. Percentage survival (mean ± SD; n = 3) of larval marble goby given rotifers (Day 0 - 30) and Artemia *nauplii (Day 21 - 30) fed with biomass of settled bacteria (sPOME-PB) or* Nannochloropsis *sp. in 5 ppt salinity water for 30 days. Initial stocking size in 25 litres = 250 larvae.*



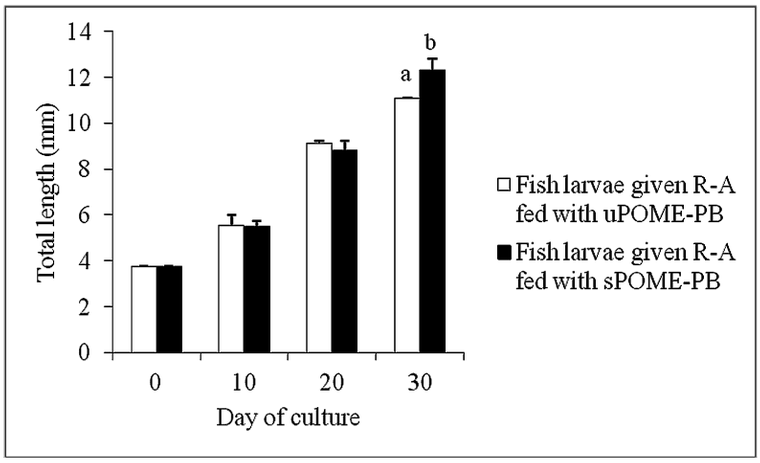
Note: [a]Indicates homogeneous group in t-test (P > 0.05).

Figure 5. Total length (mean mm ± SD; n = 9) of larval marble goby given rotifers (Day 0 - 30) and Artemia *nauplii (Day 21 - 30) fed with biomass of settled bacteria (sPOME-PB) or* Nannochloropsis *sp. in 5 ppt salinity water for 30 days. Initial stocking size in 25 litres = 250 larvae.*



Note: [a]Indicates homogeneous group in t-test (P > 0.05).

Figure 6. Percentage survival (mean ± SD; n = 3) of larval marble goby given rotifers (Day 0 - 30) and Artemia *nauplii (Day 21 - 30) fed with unsettled bacterial culture (uPOME-PB) or biomass of settled bacteria (sPOME-PB) in 5 ppt salinity water for 30 days. Initial stocking size in 25 litres = 375 larvae.*



Note: [a,b]Indicate homogeneous groups in *t*-test (P > 0.05).

Figure 7. Total length (mean mm ± SD; n = 9) of larval marble goby given rotifers (Day 0 - 30) and Artemia *nauplii (Day 21 - 30) fed with unsettled bacterial culture (uPOME-PB) or biomass of settled bacteria (sPOME-PB) in 5 ppt salinity water for 30 days. Initial stocking size in 25 litres = 375 larvae.*

9.60 mm TL at 5 ppt salinity (Senoo *et al*., 2008). Increasing the fish stocking density from 10 larvae litre^{-1} to 15 larvae litre^{-1} did not affect the larval performance. In fact, the survival and growth of larvae reared at 15 larvae litre^{-1} was significantly higher than larvae at 10 larvae litre^{-1}.

CONCLUSION

POME enriched bacteria (POME-PB) is a potential live feed for aquaculture. High rotifer density can be achieved in a short period of culture using uPOME-PB as a total rotifer diet. The significantly improved survival and growth of marble goby larvae given rotifers and *Artemia* nauplii fed with uPOME-PB or sPOME-PB have paved the way to a stable rearing technique for marble goby larvae, and perhaps, its large scale commercial production. Furthermore, another profitable commercialisation opportunity looks possible, *i.e.* the cheap production of POME enriched bacteria as feed for producing various zooplanktons and other live feed crucial to the aquaculture industry. Based on our results and projected estimates 60 litres of POME could in four days be converted to 448 g of dry sPOME-PB which could produce rotifers in 1 t of water, at a density of up to 898 rotifers ml^{-1}. The enriched rotifers could be used to feed 1.35 million larval marble goby on a daily basis.

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