Growth condition optimization for docosahexaenoic acid (DHA) production by *Moritella marina* MP-1

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ABSTRACT

The marine organism *Moritella marina* MP-1 produces the polyunsaturated fatty acid docosahexaenoic acid (DHA). While the basic metabolic pathway for DHA production in this organism has been identified, the impact of growth conditions on DHA production is largely unknown. This study examines the effect of supplemental carbon, nitrogen and salts, growth temperature and media composition and pH on DHA and biomass production and the fatty acid profile. The addition of supplemental nitrogen significantly increased the overall DHA titer via an increase in biomass production. Supplemental glucose or glycerol increased biomass production, but decreased the amount of DHA per biomass, resulting in no net change in the DHA titer. Acidification of the baseline media pH to 6.0 increased DHA per biomass. Changes in growth temperature or provision of supplemental sodium or magnesium chloride did not increase DHA titer. This organism was also shown to grow on defined minimal media. For both media types, glycerol enabled more DHA production per biomass than glucose. Combination of these growth findings into marine broth supplemented with glycerol, yeast extract and tryptone at pH 6.0 resulted in a final titer of 82±5 mg/L, a nearly 8-fold increase relative to the titer of 11±1 mg/L seen in the unsupplemented marine broth. The relative distribution of other fatty acids was relatively robust to growth condition, but the presence of glycerol resulted in a significant increase in myristic acid (C14:0) and decrease in palmitic acid (C16:0). In summary, DHA production by *M. marina* MP-1 can be increased more than 5-fold by changing the growth media. Metabolic engineering of this organism to increase the amount of DHA produced per biomass could result in additional increases in titer.

Keywords: *Moritella marina* MP-1, polyunsaturated fatty acids, membrane fluidity, polyketide, marine broth

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) such as docosahexanoic acid (DHA, 22:6(n-3)) and eicosapentaenoic acid (EPA, 20:5(n-3)), have beneficial effects on human health through their effect on membrane fluidity and contribute to many aspects of health, including but not limited to, fetal development and prevention of cancer and obesity (Chapkin et al. 2008; Connor et al. 2000; Lagarde 2008; Li et al. 2008; Siddiqui et al. 2008; Valenzuela 2009). It is also established that these compounds downregulate inflammatory pathways that are known to be activated in chronic diseases such as rheumatoid arthritis, obesity and type 2 diabetes, among others (Wall et al. 2010). It is recommended that the general public consume at least 100 mg of omega-3 fatty acids such as DHA daily, with some agencies suggesting that this dose be increased 10-fold (Nishida et al. 2004). This increasing demand for PUFA supplements is complicated by the fact that even the current demand cannot be sustainably met by fish-derived PUFAs (Jenkins et al. 2009). Together with concerns about possible socio-economic effects, such as the inability of local populations to continue to use fish as a food staple (Alder and Watson 2007) and environmental contaminants in fish-derived oils (Hites et al. 2004) there is a strong demand for non-fish-derived PUFA.

Non-fish sources of PUFA include algae, recombinant plants and bacteria. Some marine bacteria, particularly psychrophilic deep-sea bacteria, are especially intriguing. It has even been speculated that these marine bacteria are the original source of PUFAs in the marine food web (Napier 2002). Large amounts of EPA (12-37%) and DHA (10-25%) have been detected in certain marine bacteria, with a general increase in the PUFA content as a function of decreasing temperature and increasing pressure, suggesting that these fatty acids are involved in the maintenance of membrane fluidity (Delong and Yayanos 1986). Two recent rigorous studies demonstrated that EPA production by *Shewanella sp* plays a crucial role in stabilizing the

membrane structure (Usui 2012) and maintaining the appropriate membrane hydrophobicity (Nishida 2010). Among the characterized marine isolates, *Moritella marina* MP-1, originally known as *Vibrio marinus*, is particularly interesting due to the presence of DHA in its membrane lipids as a fatty acyl component. A systematic comparison of several marine bacteria showed that *M. marina* MP-1 produced more than twice as much DHA as the other 9 species examined (Morita et al. 2005a). A draft genome sequence of this organism was recently announced (Kathaurapu and Jarboe, *submitted*).

In contrast to the traditional desaturase-based PUFA biosynthesis pathway, *M. marina* MP-1 uses a distinct polyketide synthase (PKS)-type pathway that remains poorly characterized (Jenke-Kodama et al. 2005; Metz et al. 2001; Napier 2002). PKS-type pathways are important for a variety of complex natural products, such as cholesterol-lowering lovastatins (Campbell and Vederas), among others (Busch and Hertweck 2009; Calderone 2008; Shen 2003).

While the effect of growth condition on EPA production by *Shewanella sp* has been characterized (Jeong et al. 2006; Wang et al. 2009), the effect of growth conditions on DHA production by *M. marina* MP-1 remains relatively unexplored. It has been demonstrated that growth in media rich in fatty acids or supplemented with the antibiotic cerulenin increased the overall DHA titer (Morita et al. 2005a; Morita et al. 2005b). However, the current literature lacks a systematic investigation on the effect of growth media composition, nitrogen and carbon source, media pH, or growth temperature on DHA production by *M. marina* MP-1. Here, we investigate the effect of these variables on the biomass production, DHA production and fatty acid profile of *M. marina* MP-1.

MATERIALS AND METHODS

Microorganism and culture condition. *M. marina* MP-1 (ATCC 15381) was maintained on a readily available marine broth medium-2216 (BD Bioscience) at 15ºC and 150 rpm. The marine broth medium contains 5.0 g/L peptone, 1.0 g/L yeast extract, 0.1 g/L ferric citrate, 19.45 g/L sodium chloride, 5.9 g/L magnesium chloride, 3.24 g/L magnesium sulfate, 1.8 g/L calcium chloride, 0.55 g/L potassium chloride, 0.16 g/L sodium bicarbonate, 0.08 g/L potassium bromide, 34.0 mg/L strontium chloride, 22.0 mg/L boric acid, 4.0 mg/L sodium silicate, 2.4 mg/L sodium fluoride, 1.6 mg/L ammonium nitrate, 8.0 mg/L disodium phosphate. The medium was sterilized by autoclaving and pH was adjusted with 1N HCL or 1N NaOH. Supplemental yeast extract, tryptone and ammonium chloride were prepared as 200 g/L stocks and sterilized by either autoclaving (yeast extract and tryptone) or filtration (ammonium chloride). MOPS media was prepared as previously described (Wanner 1994), but modified by the addition of NaCl to a final concentration was 20 g/L, hereafter referred to as MMOPS. MMOPS media was sterilized by filtration.

Sampling and harvesting of cells. The cells were harvested by centrifugation at 4,000 rpm for 10 minutes at 4ºC. Dry mass was determined after freeze drying cells for 12 to 24 hr.

Extraction and DHA analysis. The DHA was measured as a fatty acid methyl ester (FAME), produced by fatty acid extraction of the dry cell pellet, followed by methylation and measurement by gas chromatography/mass spectrometry (Bligh and Dyer 1959; Kramer et al. 1997; Morita et al. 2005b). Briefly, total lipids were extracted from the dry pellet using a chloroform-methanol mixture and then mixed with 2.5ml of 0.37% KCl solution. The organic phase containing free fatty acids was separated and dried under nitrogen gas at 50ºC. FAMEs were made by a direct transesterfication procedure by using methanol-acetyl chloride as previously described (Kramer et al. 1997). The FAMEs suspended in hexane were quantified by GCMS (Agilent 6890/5973) using

a BPX70 capillary column (SGE, 30.0m x 0.22mm x 0.25 μm Austin, TX) (Morita et al. 2005b). Helium was used as the carrier gas. Peak areas were quantified and compounds were identified by comparison of their retention times with those of known standards. Heneicosanoic acid (C21:0) was used as an internal standard and DHA was quantified from a DHA/ISTD curve. Each biological sample was analyzed in triplicate and at least three biological replicates were analyzed for each experimental condition. Data are expressed as a mean ± standard deviation. DHA content is reported both on a per biomass basis (mg DHA per g of biomass) or as an overall titer (mg DHA/L culture broth), as calculated by the DHA per biomass multiplied by the amount of biomass per L. Statistical significance was determined by the Student's t-Test; a cut-off value of $P < 0.05$ was used.

RESULTS

Here we have investigated the impact of media composition, media pH and growth temperature on production of DHA and other fatty acids by *M. marina* MP-1. The control condition for this study was unsupplemented marine broth, initially adjusted to pH 7.0, maintained at 15^{o}C with rotary shaking at 150 rpm. In the unsupplemented broth, the DHA titer was 11±1 mg DHA/L after 96 hours of growth; this corresponded to 16±1 mg DHA/g dry cell mass (Table 1).

Supplemental glucose and glycerol increase growth but not DHA titer

While marine broth is rich in nutrients and metabolites, it is desirable to provide a supplemental carbon source to fuel production of carbon-rich DHA. Therefore, the efficacy of glucose, xylose and glycerol as supplemental carbon sources was tested at 15^{o}C (Table 1, *xylose data not shown*).

Supplemental glucose and glycerol both had a positive impact on biomass production, resulting in a roughly 6- and 3-fold increase, respectively. However, there was a negative impact on the DHA content on a per biomass basis, decreasing the DHA per dry cell mass more than 3-fold. Thus there was no significant change in the DHA titer compared to the baseline condition. No growth was observed in the presence of supplemental xylose (*data not shown*). Similar results were obtained for glucose and glycerol at 10^0C: supplemental carbon increased biomass production but there was no change in DHA titer due to the corresponding decrease in the amount of DHA per biomass (Table 1)

Supplemental nitrogen increases growth and DHA titer

Our unsupplemented marine broth contains nitrogen in the form of 5.0 g/L peptone, 1.0 g/L yeast extract and 1.6 mg/L ammonium nitrate. To test the impact of increased nitrogen availability on DHA production, an additional 10 g/L yeast extract or 10 g/L tryptone was used. As with the supplemental carbon, nitrogen supplementation resulted in increased biomass production and decreased DHA per biomass (Table 1). But unlike carbon supplementation, the magnitude of the increase in biomass was sufficient to result in an increase in the DHA titer. The effect was moderate with tryptone, but yeast extract resulted in a more than 2-fold increase. Note that the addition of 15 g/L of either nitrogen source did not significantly increase the DHA titer relative to 10 g/L (*data not shown*). When the two nitrogen sources were both added to the growth media at 10 g/L of each, there was a 5-fold increase in DHA titer relative to the baseline condition (Table 1).

Thus, while provision of supplemental organic nitrogen results in a decrease in DHA per biomass, the corresponding increase in biomass production is sufficient to increase the DHA titer by 5-fold.

DHA production is increased at moderately acidic pH

Production of DHA was found to be dependent on the cultivation pH (Table 1, Figure 1), with pH 6.0 promoting a significant increase in both DHA per biomass and DHA titer relative to pH 7.0. At pH of 5.0 the growth was drastically decreased and no measurable amount of DHA was produced. Media pH values above 7.0 did not increase DHA titer or DHA per biomass (Figure 1). Thus, decreasing the media pH from 7.0 to 6.0 significantly increases both the DHA per biomass and the DHA titer, but a further decrease to pH 5.0 has a detrimental effect on DHA production.

Defined media supports DHA production

Existing studies with MP-1 have used complex media (Morita et al. 2005a; Morita et al. 2005b). Here we have tested MOPS minimal media, modified to contain 20 g/L NaCl (MMOPS), for its ability to support *M. marina* MP-1 growth and DHA production at 10, 15 and 20^0C (Table 1). No growth was observed with glucose at 20^0C (*data not shown*). While glycerol supports a higher DHA titer in minimal media than glucose, the titer was not increased relative to unsupplemented marine broth at the same temperature.

These results demonstrate that defined minimal media can be used for growth and that glycerol is a preferable carbon source for both biomass and DHA production relative to glucose in MMOPS media.

Supplemental carbon increases temperature sensitivity

In order to determine the effect of temperature on growth and DHA production, *M. marina* MP-1 was grown in marine broth with either no carbon source or 1% (w/v) glucose or glycerol at 5, 10 and 20^0C in addition to our standard temperature of 15^0C (Table 1, Figure 2).

Both the production of biomass and DHA depends on the cultivation temperature, though there was no apparent linear trend observed in all conditions (Figure 2). No growth was observed

at 5°C for any of the conditions tested or with glucose at 20°C (*data not shown*). However, it is apparent that the presence of supplemental glucose or glycerol enhanced the temperature sensitivity of biomass production, with 15°C being the optimal cultivation temperature. At each temperature, biomass production was increased relative to the unsupplemented condition while DHA per biomass was decreased when supplemental carbon was added. Thus, we have concluded at 15^0C remains the best growth temperature for maintaining biomass production and sufficient DHA titers with a variety of carbon sources.

Supplemental salts do not increase DHA production

Given that *M. marina* MP-1's natural marine habitat is rich in salts, we tested the impact of additional sodium chloride and magnesium chloride. Neither species resulted in a significant increase of biomass production, DHA per biomass or DHA titer (Table 1).

Integrated Results

The results described above show that adding supplemental carbon or nitrogen to the standard marine broth increases biomass production by *M. marina* MP-1. Adjustment of unsupplemented media pH to 6.0 instead of the standard 7.0 significantly increases the amount of DHA per biomass. We combined these findings into three new growth conditions in marine broth at 15^0C, pH 6.0 (Table 2). Combination of just the pH and supplemental carbon results did not significantly increase the DHA titer relative to the unsupplemented pH 7.0 marine broth. However, the simultaneous provision of supplemental glycerol, yeast extract and tryptone at pH 6.0 resulted in a nearly 10-fold increase in biomass production and 8-fold increase in DHA titer (Table 1).

Extension of the pH and nitrogen supplementation results in the rich marine broth to the MMOPS defined media did not result in an increase in DHA per biomass or DHA titer (Table 2). Note that in this case nitrogen was added in the inorganic form as 10 g/L ammonium chloride. For

the case where MMOPS glucose media at pH 6.0 was supplemented with ammonium chloride, no growth was observed (*data not shown*).

Fatty acid profile changes

While profiling of other fatty acids was not quantitative, we did observe changes to the relative fatty acid profile according to growth condition (Table 3). The impact of supplemental glycerol was the most dramatic effect: for each media type and temperature tested, the relative abundance of myristic acid (C14:0) increased by roughly two-fold relative to the no supplemental carbon and/or glucose-supplemented culture while palmitic acid (C16:0) decreased roughly two-fold (Figure 3). In fact, the presence of glycerol had more influence on fatty acid profiles than either changes in temperature or changes from rich to defined media (Figure 3, Table 3). The simultaneous supplementation with yeast extract and tryptone in marine broth at 15^0C, pH 7.0 also resulted in an increase of C14:0 abundance, but not the corresponding decrease in C16:0 seen with glycerol (Table 3).

DISCUSSION

M. marina MP-1 naturally produces DHA and is an attractive biocatalyst for sustainable, safe, production of this valuable nutraceutical. Here we have investigated the impact of several growth condition parameters on both DHA and biomass production by this organism (Table 1). We found that supplemental carbon in the form of glucose or glycerol or nitrogen in the form of yeast extract or tryptone increased the amount of biomass produced. For glucose and glycerol, the corresponding decrease in the DHA per biomass meant that there was no significant change in the DHA titer. For organic nitrogen supplementation, the magnitude of the increase in biomass

production was sufficient to result in an increase in the DHA titer. While we have attributed this positive effect to the organic nitrogen found in yeast extract and tryptone, it is also possible that this result is due to a specific metabolite found in these mixtures.

The only factor that we found to increase the amount of DHA per biomass was acidification of the media to pH 6.0 instead of 7.0 (Figure 1), resulting in an increase of both DHA per biomass and biomass concentration. However, further acidification to pH 5.0 drastically decreased growth and DHA production. Growth and DHA production without supplemental carbon is largely insensitive to the temperatures that we tested. However, the presence of the supplemental carbon sources increased sensitivity to temperature, with 15^{o}C being optimal for biomass production (Figure 2). Defined minimal media can be used in place of marine broth, with glycerol supporting an increased DHA titer relative to glucose. This finding is particularly intriguing, given the availability of glycerol as a by-product of the biodiesel production process.

Combination of our findings such that supplemental carbon and nitrogen were added to marine broth acidified to pH 6.0 resulted in a 7-fold increase in DHA titer to 82 $\pm$ 5 mg/L (Table 2). This titer is 5-fold higher than the 13.7 mg/L achieved by supplementing *M. marina* culture with the antibiotic cerulenin (Morita 2005b), but drastically lower than the 4.3 g/L reported to be produced by the marine thraustochytrid *Aurantiochyrium limacinum* (Nagano 2009).

Despite our observed increase in the DHA titer, the amount of DHA produced per biomass is still significantly lower than the baseline condition. This difficulty in increasing the amount of DHA per biomass suggests tight control of DHA production by this organism. Additional increases in DHA production by *M. marina* MP-1 could be attained upon mitigation of these regulatory features. Mitigation of these regulatory features would be greatly aided by their identification and characterization.

The additional trends we observed in the overall fatty acid profile could provide some insight into this regulation. Most intriguing was the fact that glycerol has a drastic impact on the relative abundance of myristic (C14:0) and palmitic (C16:0) acids (Figure 3). The magnitude of the change in the presence of glycerol is larger than that seen in response to temperature changes, media type or the presence of supplemental glucose. Given that DHA production by this organism is presumably a means of controlling membrane fluidity, it is reasonable that the membrane composition is sensitive to environmental conditions. EPA content in *Shewanella marinintestina* is known to affect the entry of hydrophobic compounds into the bacterial cell (Nishida 2010). The octanol/water partition coefficient for glycerol is known to be an order of magnitude larger than that of glucose at room temperature (Wolosin 1978, Stevens 1988). The difference in the hydrophobicity of these two compounds could explain their differing effect on the membrane composition. However, the mechanism for sensing and controlling the composition remains unknown.

In summary, modification of a few key growth parameters can result in a drastic increase in DHA production by this organism. Similar studies have been performed on the DHA-producing fungal species *Thraustochyrium aureum* (Hur 2002, Ge 2011) and marine thraustochytrid *A. limacinum* (Nagano 2009) found quite different patterns from those that we have observed for *M. marina*. For example, Hur *et al* found that glucose supplementation had no impact on DHA content per biomass, while we found that DHA content per biomass decreased in the presence of glucose. Hur *et al* also concluded that a low temperature decreased cell growth but increased the DHA content per biomass; we did not observe either of these trends in our analysis. These differences most likely reflect the different natural histories, and thus different regulatory structures, for controlling DHA production. Note that Lee *et al*'s study of *Escherichia coli* engineered for EPA

production did observe a negative relationship between EPA production and glucose supplementation (Lee 2006). Given that this engineered *E. coli* strain uses a PKS type pathway similar to the one present in *M. marina*, it is not surprising that its response to glucose availability would be more similar to *M. marina* than to *T. areum*.

Future work aimed at identifying and characterizing control of DHA production could enable further increases in production of this valuable compound and provide insight into the regulatory pathways that control its production. Such findings could have a broader impact on the production of fatty acids, and other hydrophobic compounds, as biofuels and biorenewable chemicals.

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REFERENCES

Alder J, Watson R (2007) Fisheries globalization: fair trade or piracy? In: Taylor WW, Schechter MG, Wolfson LG (eds) Globalization: Effects on Fisheries Resources. Cambridge University Press, New York, pp 47-74

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37(8):911-7

Busch B, Hertweck C (2009) Evolution of metabolic diversity in polyketide-derived pyrones: Using the non-colinear aureothin assembly line as a model system. Phytochem 70(15-16):1833-1840 doi:10.1016/j.phytochem.2009.05.022

Calderone CT (2008) Isoprenoid-like alkylations in polyketide biosynthesis. Nat Product Rep 25(5):845-853 doi:10.1039/b807243d

Campbell CD, Vederas JC Biosynthesis of Lovastatin and Related Metabolites Formed by Fungal Iterative PKS Enzymes. Biopolym 93(9):755-763 doi:10.1002/bip.21428

Chapkin RS, Seo JM, McMurray DN, Lupton JR (2008) Mechanisms by which docosahexaenoic acid and related fatty acids reduce colon cancer risk and inflammatory disorders of the intestine. Chem Phys Lipids 153(1):14-23 doi:10.1016/j.chemphyslip.2008.02.011

Connor SL, Zhu N, Anderson GJ, Hamill D, Jaffe E, Carlson J, Connor WE (2000) Cheek cell phospholipids in human infants: a marker of docosahexaenoic and arachidonic acids in the diet, plasma, and red blood cells. Am J Clin Nutr 71(1):21-27

Delong EF, Yayanos AA (1986) Biochemical function and ecological significance of novel bacterial lipids in deep-sea prokaryotes. Appl Environ Microbiol 51(4):730-737

Hites RA, Foran JA, Carpenter DO, Hamilton MC, Knuth BA, Schwager SJ (2004) Global assessment of organic contaminants in farmed salmon. Science 303(5655):226-229

Hur Byung-Ki CD-W, Kim Ho-Jung, Park Chun-Ik, Suh Hyung-Joon (2002) Effect of culture conditions on growth and production of docosahexaenoic acid (DHA) using *Thraustochytrium aureum ATCC 34304*. Biotechnol Bioprocess Eng 7(1):6

Jenke-Kodama H, Sandmann A, Muller R, Dittmann E (2005) Evolutionary implications of bacterial polyketide synthases. Mol Biol Evol 22(10):2027-2039 doi:10.1093/molbev/msi193

Jenkins DJA, Sievenpiper JL, Pauly D, Sumaila UR, Kendall CWC, Mowat FM (2009) Are dietary recommendations for the use of fish oils sustainable? Can Med Assoc J 180(6):633-637 doi:10.1503/cmaj.081274

Jeong YS, Song SK, Lee SJ, Hur BK (2006) The growth and EPA synthesis of *Shewanella oneidensis* MR-1 and expectation of EPA biosynthetic pathway. Biotechnol Bioprocess Eng 11(2):127-133

Kramer JK, Fellner V, Dugan ME, Sauer FD, Mossoba MM, Yurawecz MP (1997) Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. Lipids 32(11):1219-28

Lagarde M (2008) Docosahexaenoic acid: Nutrient and precursor of bioactive lipids. Eur J Lipid Science Technol 110(8):673-678 doi:10.1002/ejlt.200800087

Lee SJ, Jeong YS, Kim DU, Se JW, Hur BK (2006) Eicosapentaenoic acid (EPA) biosynthetic gene cluster of *Shewanella oneidensis MR-1*: Cloning, heterologous expression, and effects of temperature and glucose on the production of EPA in *Escherichia coli*. Biotechnol Bioprocess Eng 11(6):510-515

Li JJ, Huang CJ, Xie D (2008) Anti-obesity effects of conjugated linoleic acid, docosahexaenoic acid, and eicosapentaenoic acid. Mol Nutr Food Res 52(6):631-645 doi:10.1002/mnfr.200700399

Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science 293(5528):290-293

Morita N, Ichise N, Yumoto I, Yano Y, Ohgiya S, Okuyama H (2005a) Cultivation of microorganisms in the cultural medium made from squid internal organs and accumulation of polyunsaturated fatty acids in the cells. Biotechnol Lett 27(13):933-941 doi:10.1007/s10529-005-7187-3

Morita N, Nishida T, Tanaka M, Yano Y, Okuyama H (2005b) Enhancement of polyunsaturated fatty acid production by cerulenin treatment in polyunsaturated fatty acid-producing bacteria. Biotechnol Lett 27(6):389-93 doi:10.1007/s10529-005-1532-4

Nagano N, Taoka Y, Honda D, Hayashi M (2009) Optimization of culture conditions for growth and docosahexaenoic acid production by a *marine thraustochytrid*, *Aurantiochytrium limacinum* mh0186. J Oleo Sci 58(12):623-8

Napier JA (2002) Plumbing the depths of PUFA biosynthesis: a novel polyketide synthase-like pathway from marine organisms. Trends Plant Science 7(2):51-54

Nishida C, Uauy R, Kumanyika S, Shetty P (2004) The Joint WHO/FAO Expert Consultation on diet, nutrition and the prevention of chronic diseases: process, product and policy implications. Public Health Nutr 7(1A):245-250 doi:10.1079/PHN2003592

Nishida T, Hori R, Morita N, Okuyama H (2010) Membrane eicosapentaenoic acid is involved in the hydrophobicity of bacterial cells and affects the entry of hydrophilic and hydrophobic compounds. FEMS Microbiol Lett 306:91-96. doi: 10.111/j.1574-6968.2010.01943x

Shen B (2003) Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. Curr Opin Chem Biol 7(2):285-295 doi:10.1016/s1367-5931(03)00020-6

Siddiqui RA, Harvey K, Stillwell W (2008) Anticancer properties of oxidation products of docosahexaenoic acid. Chem Phys Lipids 153(1):47-56 doi:10.1016/j.chemphyslip.2008.02.009

Stevens PJC, Baker EA, Anderson NH (1988) Factors affecting the foliar absorption and redistribution of pesticides. 2. Physicochemical properties of the active ingredient and the role of the surfactant. Pestic Science 24(1):31-53.

Usui K, Hiraki T, Kawamoto J, Kurihara T, Nogi Y, Kato C, Abe, F (2012) Eicosapentaenoic acid plays a role in stabilizing dynamic membrane structure in the deep-sea piezophile *Shewanella violacea*: a study employing high-pressure time-resolved fluorescence anisotropy measurement. Biochim Biophys Acta 1818:574-583 doi: 10.1016/j.bbamem.2011.10.010.

Valenzuela A (2009) Docosahexaenoic acid (DHA), an essential fatty acid for the proper functioning of neuronal cells: their role in mood disorders. Grasas Y Aceites 60(2):203-212 doi:10.3989/gya.085208

Wall R, Ross RP, Fitzgerald GF, Stanton C (2010) Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev 68(5):280-9 doi:10.1111/j.1753-4887.2010.00287.x

Wang F, Xiao X, Ou HY, Gai YB, Wang FP (2009) Role and Regulation of Fatty Acid Biosynthesis in the Response of *Shewanella piezotolerans* WP3 to Different Temperatures and Pressures. J Bacteriol 191(8):2574-2584 doi:10.1128/jb.00498-08

Wanner BL (1994) Gene expression in bacteria using TnphoA and TnphoA' elements to make and switch phoA gene, lacZ (op) and lacZ (pr) fusions. In: Adolph KW (ed) Methods Mol Genet. vol 3. Academic, Orlando, FL, pp 291-310

Wolosin JM, Ginsburg H, Lieb WR, Stein WD (1978) Diffusion within egg lecithin bilayers resembles that within soft polymers. J Gen Physiol 71(1):92-100.

Yang Ge LY, Gao Ming (2011) Effects Of pH And Aeration On The Production Of docosahexaenoic acid By *Thraustochytrium Aureum* In Controlled Batch Fermentor Cultures. Adv Mater Res 183-185:5 oi:doi:10.4028/www.scientific.net/AMR.183-185.50

Figure Legends:

Figure 1: Effect of initial media pH on biomass and DHA production. Cells were grown in unsupplemented marine broth at 15^{o}C and 150 rpm for 96 hours. Data from at least 3 biological replicates are expressed as mean ± one standard deviation.

Figure 2: Effect of temperature on (A) DHA per biomass, (B) biomass production and (C) DHA titer. Cells were grown in marine broth containing either no supplemental carbon or 1% (w/v) glucose or glycerol at pH 7.0, 150rpm for 96 hours. Data from 3 biological replicates are expressed as mean ± one standard deviation. No growth was observed for the marine broth with glucose at 20^{o}C.
* indicates at P value less than 0.05 relative to the baseline condition at the same temperature

Figure 3: Effect of supplemental glycerol on the relative abundance of myristic (C14:0) and palmitic (C16:0) fatty acid methyl esters. Cells were grown at pH 7.0, 150rpm for 96 hours. Data from at least 3 biological replicates are expressed as mean ± one standard deviation.