Research article

Two-level factorial analysis of the effect of fructose on DHA biosynthetic capacity of *Aurantiochytrium* sp. SW1

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**ABSTRACT**

Thraustochytrids are getting increasingly popular due to their high potential role as alternative producers of the high-valued ω-3 polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA). While most thraustochytrids prefer glucose as the major carbon source, few strains have been reported to prefer fructose. One such strain is *Aurantiochytrium* sp. SW1. In this study, the effect of fructose on DHA accumulation by SW1 was investigated using a two-level full factorial design. Besides, biomass, lipid and DHA accumulation profiles of SW1 cultivated in fructose and glucose media were compared. Results revealed that fructose has a very significant positive effect on the volumetric DHA content. Meanwhile, its involvement in affecting DHA biosynthetic capacity, though significant, is not very profound. It was also found that when cultivated in fructose medium, SW1 had a less steep log phase compared to that of glucose medium. However, after 48h of cultivation, biomass and lipid accumulation in fructose medium outweighed the other. Volumetric DHA content in fructose medium at 96h was 11% higher than that of glucose medium. Overall, fructose was found to be a more suitable substrate for biomass, lipid and DHA accumulation in SW1 compared to the conventional source, glucose.

1. Introduction

Omega-3 fatty acids, in the past decades, have been widely interrelated to healthy aging due to the many fundamental roles they play in human health and development. Docosahexaenoic acid (DHA) is an important member of the ω-3 family. It is an essential component in the brain, skin and eyes [1,2]. This fatty acid is typically acquired from fatty fish such as salmon, mackerel and swordfish, crustaceans such as crab and spiny lobster, as well as mollusks such as scallop and oyster [3].

Ever since the introduction of the concept of “designer oils” [4], bioproduction of DHA from microalgae has gained great interest over the conventional method of obtaining it from seafood. The idea of acquiring DHA from microalgae instead of seafood-based sources is advantageous. Besides better purification potential, microalgal DHA is usually free from chemical contamination [5]. Unlike depleting fish stock that is incapable to provide adequate supply for the continually increasing market demand [6], bioproduction of DHA from microalgae promises a continuous supply that can be adjusted depending on the demand.

Thraustochytrids are unicellular heterokont microalgae, having an extensive geographical distribution from the polar to tropical regions, inhabiting various environments like mangrove sediments, estuaries and deep-sea ecosystems [7]. Lacking chloroplasts, they are unable to perform photosynthesis but possess excellent DHA synthesis capabilities which make them considered to be a primary DHA producer in marine environments [8]. Thraustochytrids have suitable properties for the industrial production of DHA, i.e., they can be mass cultured using a jar fermenter without sunlight, and they can accumulate large amounts of DHA in well-developed intracellular lipid droplets.

Several strains of thraustochytrids have been reported to produce high biomass, containing large amounts of DHA-rich lipid [9]. One such thraustochytrid is *Aurantiochytrium* sp. SW1, which has been reported to accumulate up to half of its biomass as lipid, containing 40-60% DHA from the total fatty acids. This isolate, unlike most other DHA-producing thraustochytrids, prefers fructose over glucose for biomass, lipid and DHA accumulation [10]. Utilization of this unique characteristic of SW1 is expected to lead to betterment in overall DHA productivity. As previous studies only report on the basis of general production parameters such as concentration and yield, in this study, we intend to define the significance of fructose as the major carbon source for DHA production using a two-level full factorial design. Compared to the conventional ‘one factor at a time’ (OFAT) method, the factorial design offers a wider inductive basis, where it covers a broader space from which inferences
could be drawn. Besides, analysis using this method enables the determination of the magnitude of the effect of fructose on its own as well as its interaction with other medium components. This provides the key to understanding the overall effects [11] of all medium components on DHA production in SW1.

2. Materials and methods

2.1. Microorganism and culture conditions

*Auxanotrichum* sp. SW1 (GenBank: KF500513, UNICC UPM-WDCM 988: UPMC 963) was obtained from the Microbial Physiology Laboratory, School of Biosciences and Biotechnology, Universiti Kebangsaan Malaysia. Glucose (60 g/L) was replaced with fructose (60 g/L) for the preparation of seed cultures involving production cultures containing fructose. Cultivation was carried out in 500 mL Erlenmeyer flasks at 30°C.

2.2. Experimental design

A two-level full factorial design was employed to investigate the effects of fructose and other components present in the production medium. Design expert software (DOE; version 10.0, Stat-Ease, USA) was used to design the experiments. The ranges of the medium components are shown in Table 1. The ranges were chosen based on preliminary experiments (data not shown).

The design of the experiment together with the response values are shown in Table 2. The design contained a duplicate for each run, summing up to a total run number of 32. The confidence level was set at 95%.

2.3. Analytical methods

Culture conditions and analytical methods for the determination of biomass, lipid, and DHA concentrations are as described in our previous work [12].

3. Results

In our previous studies, experimentations to investigate the ability of SW1 to utilize different carbon sources showed that fructose is the most preferred sugar for biomass, lipid, and DHA accumulation based on the levels of DHA concentration and yield achieved [10]. Similar results were observed by Chatdumrong et al. [13] where *S. limacinum* BR2.1.2 produced an enhanced amount of DHA when fructose was used as a major carbon source replacing glucose. Therefore, in this study, a two-level full factorial design was employed to further establish the significance of fructose on DHA accumulation with a particular emphasis on its interactions with other medium components. For better evaluation of the impact of fructose, DHA accumulation was represented as volumetric content (g/L culture) as well as DHA biosynthetic capacity of the cells (% g/g dry biomass). Results of the experiment are shown in Table 2.

3.1. Effects of fructose on volumetric content (g/L) of DHA

The effect of fructose, as well as other factors on the volumetric DHA production, was initially visualized by the significance of each coefficient, indicated by the F and p values from the analysis of variance (ANOVA) data (Table 3). Values of ‘Prob > F’ less than 0.005 indicate that the model is significant and vice versa. Based on Table 3, the p-value of the model was (p < 0.0001) and the F-value was large (562.77) implying that the model was significant to interpret the data. "Lack of Fit p-value" was 0.8475 indicating the Lack of Fit was not significant relative to the pure error. The ANOVA showed that all the above-listed factors and interactions, except factor C (MSG), are highly significant, with a probability value less than 0.0001.

Besides, it is important to determine the relative effect of fructose and its interaction with other variables to determine the extent of its participation in the effect of the volumetric output of DHA by SW1. Thus, the changes in response values or term as “standardized effects” were demonstrated in Figure 1. This value functions as an indicator of the magnitude of effect contributed by a factor as well as its direction, whether it is a positive or negative effect. The summary of the effects of all medium components and their interactions on the volumetric content of DHA is illustrated in Figure 1.

Figure 1 notably shows that factors A (fructose) and B (yeast extract), as well as their interaction, exert very significant positive effects on the DHA content compared to other factors. Similarly, interactions between AC (fructose-MSG) and CD (MSG-sea salt) affect DHA content on a considerable negative scale, whereas AD (fructose-sea salt) and BD (yeast extract-sea salt) contribute small negative effects. As for 3-factor interactions, BCD (yeast extract-MSG-sea salt) exert visible negative effect, meanwhile ABC (fructose-yeast extract-MSG), ABD (fructose-yeast extract-sea salt) and ACD (fructose-MSG-sea salt) exerts positive effect. Overall, based on Figure 1, fructose corresponds to three-quarters of the four most impactful factors (A, B, AB and ACD). This indicates the significant positive role played by fructose among the rest of the medium components in boosting the volumetric DHA content of SW1. This is due to fructose, being the carbon source, is a critical compound in the biosynthesis of fatty acids.

3.2. Effects of fructose on DHA biosynthetic capacity (% g/g biomass) of SW1

ANOVA results on the effects of fructose on DHA biosynthetic capacity (% g/g biomass) of SW1 were indicated in Table 4. The result showed that factors B, AB, AC, AD, BD, CD, ACD and BCD are the highly significant factors with probability value of less than 0.0001; meanwhile, factors A, C and ABD score probability value of 0.0181, 0.0007 and 0.0025 respectively. Even though the role of fructose in promoting DHA biosynthetic capacity of SW1 is less prominent compared to that of DHA content, it can still be concluded that fructose does have a significant positive effect based on its involvement in the very significant interactions, AB and ACD.

Similarly, the standardized effects of the medium components and their interactions on the DHA biosynthetic capacity of SW1 were also evaluated (Figure 2). In contrast to volumetric DHA content discussed in the previous section, the biosynthetic capacity, which reflects the amount of DHA capable to be synthesized per gram of biomass, is affected approximately equally in both positive and negative directions by the medium components and their interactions (Figure 2).

Fructose, while appearing to be a negative factor in contrast to its effect on the volumetric content of DHA, exerts a very small effect on the DHA biosynthetic capacity of SW1. However, its interaction with yeast extract (AB) and MSG-sea salt (ACD) have a profound positive effect.

| Factors | Low level (-1) (g/L) | High level (+1) (g/L) |
|---------|---------------------|----------------------|
| A: Fructose | 0 | 60 |
| B: Yeast extract | 0 | 2 |
| C: MSG | 0 | 8 |
| D: Sea salt | 0 | 6 |
Table 2. Experimental design and response values for two-level factorial analysis.

| Std order | Fructose | Yeast extract | MSG | Sea salt | DHA (g/L) Predicted | Actual | DHA biosynthetic capacity (% g/g biomass) Predicted | Actual |
|-----------|----------|---------------|-----|----------|---------------------|--------|-----------------------------------------------|--------|
| 1         | -1       | -1            | -1  | -1       | 0.44                | 0.46   | 15.80                                         | 15.81  |
| 2         | -1       | -1            | -1  | -1       | 0.46                | 0.46   | 15.75                                         |        |
| 3         | 1        | -1            | -1  | -1       | 1.61                | 1.45   | 24.03                                         | 25.35  |
| 4         | 1        | -1            | -1  | -1       | 1.74                |        | 22.75                                         |        |
| 5         | -1       | 1             | -1  | -1       | 0.22                | 0.19   | 7.97                                          | 7.88   |
| 6         | -1       | 1             | -1  | -1       | 0.20                |        | 8.10                                          |        |
| 7         | 1        | 1             | -1  | -1       | 3.28                | 3.15   | 29.03                                         | 29.15  |
| 8         | 1        | 1             | -1  | -1       | 3.08                |        | 28.87                                         |        |
| 9         | -1       | -1            | 1   | -1       | 1.17                | 1.07   | 24.74                                         | 23.41  |
| 10        | -1       | -1            | 1   | -1       | 1.23                |        | 26.11                                         |        |
| 11        | 1        | -1            | 1   | -1       | 1.15                | 1.19   | 18.11                                         | 18.00  |
| 12        | 1        | -1            | 1   | -1       | 1.14                |        | 18.18                                         |        |
| 13        | -1       | 1             | 1   | -1       | 2.00                | 2.12   | 30.47                                         | 31.83  |
| 14        | -1       | 1             | 1   | -1       | 1.91                |        | 29.07                                         |        |
| 15        | 1        | 1             | 1   | -1       | 3.04                | 3.54   | 27.00                                         | 26.92  |
| 16        | 1        | 1             | 1   | -1       | 3.27                |        | 27.12                                         |        |
| 17        | -1       | -1            | -1  | 1        | 1.36                | 1.38   | 20.28                                         | 20.54  |
| 18        | -1       | -1            | -1  | 1        | 1.31                |        | 19.97                                         |        |
| 19        | 1        | -1            | -1  | 1        | 1.15                | 1.19   | 11.60                                         | 11.63  |
| 20        | 1        | -1            | -1  | 1        | 1.15                |        | 11.62                                         |        |
| 21        | -1       | 1             | -1  | 1        | 1.19                | 1.23   | 32.59                                         | 32.54  |
| 22        | -1       | 1             | -1  | 1        | 1.18                |        | 32.69                                         |        |
| 23        | 1        | 1             | -1  | 1        | 3.85                | 3.45   | 32.29                                         | 32.50  |
| 24        | 1        | 1             | -1  | 1        | 3.26                |        | 32.04                                         |        |
| 25        | -1       | -1            | 1   | 1        | 1.09                | 1.17   | 23.78                                         | 22.24  |
| 26        | -1       | -1            | 1   | 1        | 1.04                |        | 25.37                                         |        |
| 27        | 1        | -1            | 1   | 1        | 1.18                | 1.12   | 12.08                                         | 12.71  |
| 28        | 1        | -1            | 1   | 1        | 1.20                |        | 11.40                                         |        |
| 29        | -1       | 1             | 1   | 1        | 0.69                | 0.73   | 16.25                                         | 16.59  |
| 30        | -1       | 1             | 1   | 1        | 0.62                |        | 15.86                                         |        |
| 31        | 1        | 1             | 1   | 1        | 3.84                | 3.91   | 22.62                                         | 22.09  |
| 32        | 1        | 1             | 1   | 1        | 3.81                |        | 23.19                                         |        |

Table 3. ANOVA on the effect of fructose and other factors on volumetric content (g/L) of DHA.

| Source | Sum of Squares | df | Mean Square | F Value | p-value | Prob > F |
|--------|----------------|----|-------------|---------|---------|----------|
| Model  | 87.39          | 12 | 7.28        | 562.77  | <0.0001 | (significant) |
| A-Frc  | 31.80          | 1  | 31.80       | 2457.50 | <0.0001 |          |
| B-YE   | 24.33          | 1  | 24.33       | 1879.84 | <0.0001 |          |
| C-MSG  | 6.125E-004     | 1  | 6.125E-004  | 0.047   | 0.8301  |          |
| D-Salt | 0.30           | 1  | 0.30        | 23.51   | 0.0001  |          |
| AB     | 24.12          | 1  | 24.12       | 1863.70 | <0.0001 |          |
| AC     | 1.45           | 1  | 1.45        | 112.33  | <0.0001 |          |
| AD     | 0.83           | 1  | 0.83        | 64.30   | <0.0001 |          |
| BD     | 0.71           | 1  | 0.71        | 54.72   | <0.0001 |          |
| CD     | 1.58           | 1  | 1.58        | 122.43  | <0.0001 |          |
| ABC    | 0.33           | 1  | 0.33        | 25.67   | <0.0001 |          |
| ACD    | 1.13           | 1  | 1.13        | 86.94   | <0.0001 |          |
| BCD    | 0.81           | 1  | 0.81        | 62.32   | <0.0001 |          |
| Residual | 0.25          | 19 | 0.013       | 0.27    | 0.8475  | (Not significant) |
| Lack of Fit | 0.012       | 3  | 3.921E-003  | 0.27    | 0.8475  | (Not significant) |
| Pure Error | 0.23         | 16 | 0.015       |         |         |          |
| Cor Total | 87.63         | 31 |             |         |         |          |
Figure 1. Effects of medium components and their interaction on the volumetric content of DHA.

Figure 2. Effects of medium components and their interactions on the DHA biosynthetic capacity of SW1.
Lipid-G
Biomass-F
DHA biosynthetic capacity-F
DHA-F

enhance and engineer the lipid production of these organisms [14,15]. Of improve market prospects. Many different strategies are utilized to production of DHA, thraustochytrids are studied extensively in order to

4. Discussion

Based on the chart, cultures which had fructose, though with a marginally less steep log phase (24–48h), results in noticeably higher biomass and lipid accumulation at 72h onwards. As for volumetric DHA content, 3.84 g/L was achieved using fructose, which was 11% higher than that of glucose. However, only negligible differences were observed in terms of DHA biosynthetic capacity between the two cultures throughout the cultivation period.

Therefore the increment in the volumetric DHA content was a result of the increase in biomass in the culture which was supplied with fructose as the major carbon source. These results are in good agreement with the observations in the two-level factorial experiments discussed above.

3.3. Growth, lipid and DHA accumulation profiles of SW1

Figure 3 illustrates the biomass, lipid and DHA accumulation profiles of SW1 cultivated separately in media containing glucose and fructose. Based on the chart, cultures which had fructose, though with a marginally less steep log phase (24–48h), results in noticeably higher biomass and lipid accumulation at 72h onwards. As for volumetric DHA content, 3.84 g/L was achieved using fructose, which was 11% higher than that of glucose. However, only negligible differences were observed in terms of DHA biosynthetic capacity between the two cultures throughout the cultivation period.

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4. Discussion

Being a very attractive and promising material for the sustainable production of DHA, thraustochytrids are studied extensively in order to improve market prospects. Many different strategies are utilized to enhance and engineer the lipid production of these organisms [14,15]. Of such, impacts of the composition of growth media are vastly studied and literature is profuse about it [16]. Fructose, along with other mono-saccharides, especially glucose, as well as polysaccharides such as glycerol, is assimilated well by various strains of Aurantiocythrum and Schizochytrium. Nevertheless, the assimilation capacities of each substrates differ among strains and few strains have been reported to have less preference over fructose compared to the other substrates. Table 5 lists a few examples of thraustochytrids and their fructose assimilation capabilities.

In our previous studies, intended to evaluate the use of glucose as the major carbon source for DHA accumulation, it was found that DHA concentration in total lipid was only affected significantly by glucose-yeast extract and glucose-MSG interactions. In line with this study, the single factor and interaction of fructose-yeast extract were also shown to poses a strong impact on the volumetric DHA production of SW1 (Figure 1). This could be related to their vital functions in the lipid biosynthesis pathway and growth. An increment in the concentration of carbon sources (particularly fructose in this study) is known to enhance lipid production in all oleaginous microorganisms including thraustochytrids as it provides excess continuous carbon flux in the central carbon backbone which then enters the lipid biosynthesis pathway upon nitrogen limitation is reached [28]. On the other hand, yeast extract provides essential vitamins particularly B complex which is vital for cell growth, thus directly correlates to its positive effects on the DHA volumetric content by positively influencing biomass concentration. Never-theless, in contrast to the previous study, it was found that fructose-MSG interaction has a negative effect on DHA concentration [29]. Similarly, another study aimed at medium optimization for SW1 revealed that glucose, neither as a single factor nor in interaction, affect DHA concentration significantly [12]. This clearly shows that improvement of DHA accumulation by SW1 can better be achieved using fructose, compared to the most conventional substrate, glucose. Furthermore, a positively significant 3-factor interaction of fructose-MSG-sea salt (ACD) observed in the current study corresponds with the previous finding, where glucose-MSG interaction was found to positively affect DHA concentration [29].

While DHA biosynthetic capacity serves as a good indicator of the capability of the cells to synthesize DHA, it is not always a definitive indicator for DHA production. Cultivation that results in high biomass concentration but with low biosynthetic capacity can still result in more products compared to those with low biomass and high biosynthetic capacity. Therefore, to achieve optimal production, it is important to consider both volumetric DHA content together with biosynthetic capacity. Since fructose and its interaction with yeast extract (AB) were found to have a significant positive effect on both volumetric DHA content and DHA biosynthetic capacity, it can be concluded that manipulation of these two factors is crucial in any attempts to enhance the production.

Having a better preference for fructose compared to glucose for both biomass and DHA accumulation is less common among the known.
thraustochytrids. As listed in Table 3, SW1, *Aurantiochytrium* sp. YLH70 [25], *S.limacinum* BR2.1.2 [13] and *Aurantiochytrium* sp. ZJWZ-7 [27] are among the few reported strains that possess this unique preference. This unique attribute possibly due to a more active fructose transport system within the membrane of these Thraustochytrids, as what was observed in the fructose-preferred *Corynebacterium glutamicum* which showed that fructose was predominantly catabolized through phosphoenolpyruvate-dependent phosphotransferase systems, resulting in a major entry of fructose via fructose 1,6-bisphosphate for the production of dihydroxyacetone [30]. Similar mechanisms may be involved in the metabolism of fructose by SW1 and the other fructose-preferred thraustochytrids, but, further work needs to be conducted to confirm the claim. Besides, it could be also explained by reflecting the metabolism of the sugars in which the first reaction in fructose metabolism is the direct formation of fructose 1-phosphate by phosphoenolpyruvate (PEP) while the metabolism of glucose required an extra step in which glucose needs to be converted into glucose 6-phosphate first and then to fructose 6-phosphate by glucose-6-phosphate isomerase with the expense of one ATP molecule. Thus, utilizing fructose as carbon sources require less energy and steps in comparison to glucose, which may result in higher fructose in intake as compared to glucose [31,32]. This will then led to excess continuous carbon flux which enters the lipid biosynthesis pathway upon cessation of growth as a nitrogen-limited condition is reached, explaining the higher DHA production achieved in this study. However, no reports are available on further studies involving fructose as a major or supplemental carbon source. This can be related to the aspect of cost, where fructose is generally costlier compared to glucose. Nevertheless, further investigations on the fructose assimilative capabilities of some thraustochytrids could lead to new discoveries and possibilities in this field.

In conclusion, fructose serves as a positively significant carbon source for DHA accumulation by *Aurantiochytrium* sp. SW1 and its effects are more profound than that of glucose. Therefore, incorporation of fructose and/or fructose containing substrates in growth media could result in better DHA productivities.

**Declarations**

**Author contribution statement**

Vidyah Manikan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yusuf Nazir: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Aidil Abdul Hamid: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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**Data availability statement**

Data included in article/ supp. material/referenced in article.

**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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