

Research Paper

Expression analysis for genes involved in arachidonic acid biosynthesis in *Mortierella alpina* CBS 754.68

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Abstract

The time courses for production of fungal biomass, lipid, phenolic and arachidonic acid (ARA) as well as expression of the genes involved in biosynthesis of ARA and lipid were examined in *Mortierella alpina* CBS 754.68. A significant increase in the arachidonic acid content in lipids that coincided with reduced levels of lipid was obtained. Reduced gene expression occurred presumably due to the steady reduction of carbon and nitrogen resources. However, these energy resources were inefficiently compensated by the breakdown of the accumulated lipids that in turn, induced up-regulated expression of the candidate genes. The results further indicated that the expression of the GLELO encoding gene is a rate-limiting step in the biosynthesis of ARA in the early growth phase.

Key words: *Mortierella alpina*, arachidonic acid, lipid, gene expression, real time PCR.

Introduction

Arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid, ARA), a long chain polyunsaturated fatty acid (PUFA) of the n-6 class, plays important roles in the structure and function of biological membranes (Jang *et al.*, 2005). Lower fungi of Zygomycetes especially *Mortierella alpina* were served as rich sources of PUFAs (Sing and Ward, 1997).

Attempts to describe the biosynthetic pathways of PUFA have been continued for a long time, but it is lately that the genes regulating key steps in the biosynthesis have been identified (Shimizu *et al.*, 2003). Genes that are involved in ARA and lipid biosynthesis are classified into three groups: desaturase, elongase and malic encoding genes. Malic enzyme is the providing NADPH for fatty acid biosynthesis in *Mortierella alpina* (Zhangy and Ratledge, 2008). ARA is produced through the n-6 pathway, which

involves $\Delta 9$, $\Delta 12$, $\Delta 6$ and $\Delta 5$ desaturases and elongases (GLELO and MALCE1). The elongases, GLELO and MALCE1, have been reported to catalyse the elongation of gamma linolenic acid (18:3n-6) to dihomo- γ -linolenic acid (20:3n-6) and C16 to C18, respectively (Sakuradani *et al.*, 2009).

In the previous report, the authors proposed a growth model capable of predicting maximum ARA production (that is up to 55% of total lipid) during 10-day growth using an optimized medium containing (g/L) 50 glucose and 20 fat-free soybean meal (Samadlouie *et al.*, 2012). In the present study, the time courses of fungal growth, phenolic, lipid, ARA accumulation and substrate consumption were investigated. In addition, the expression levels of genes encoding desaturase ($\Delta 5$, $\Delta 6$, $\Delta 9$, $\Delta 12$), elongase, and malic enzyme involving in ARA and lipid biosynthesis under optimal conditions was investigated.

Materials and Methods

Microorganism, inoculum preparation and culture conditions

Mortierella alpina CBS 754.68 was purchased from Centraalbureau Schimmelcultures (CBS, the Netherlands). The seed culture medium contained (g/L): glucose 30 and yeast extract 7. The seed culture of 100 mL was incubated at 25 °C for two days in a gyratory shaker at 185 rpm. The fermentation medium was inoculated with 5% (v/v) of the mycelium suspension of the seed culture. Fermentation was carried out at 21 °C; pH 6 for ten days with glucose (50 g/L) and soy bean meal (20 g/L) as carbon and nitrogen resources, respectively.

Analytical methods

The dinitrosalicylic acid (DNS) method was used to determine the reducing sugar concentration (Miller, 1959). Protein concentration was assayed using the Lowry's method (Lowry *et al.*, 1951). The dry weight of biomass, total phenolic content, total lipids and content of ARA were determined as described in previous publications (Folch *et al.*, 1957; Waterhouse, 2002; Jang *et al.*, 2005; Metcalfe *et al.*, 1996).

RNA extraction, reverse transcription and Real-time PCR

Total RNA was extracted using the RNA Kit according to the manufacturer's instructions (Invitrogen) The NCBI database was scanned for genes encoding for fatty acid desaturases and elongases. The hints were aligned and the conserved regions were used for primer designing. For

malic enzyme encoding gene (GenBank ID: DQ973624.1), only one hint was found which used for primer designing. PCR Primers for mentioned genes and the Housekeeping Gene (actin) were designed using the Gene Runner Design software. The primers for all genes are given in Table 1. cDNA was synthesized using the cDNA synthesis kit (Invitrogen) using oligo (dT) as primers according to the manufacturer's instructions. Expression analysis of genes involving in ARA and lipid biosynthesis was carried out using Real-time PCR. The Real-time PCR mixtures containing cDNA and each primer were heated at 95 °C for 15 min and then subjected to 40 cycles consisting of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s, and finally for an extension of 10 min at 72 °C. The expression levels of target genes were normalized based on actin encoding gene (Sakuradani *et al.*, 2009) ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$). Since the total number of cycles in the Real-Time PCR was 40, for easier interpretation, the ΔCt was converted into 40- ΔCt (Czechowski *et al.*, 2004).

Results and Discussion

Dynamics of substrate consumption and biomass, phenolic, lipid and ARA production

Reducing sugars consumed slowly in the first days of cultivation followed by quick consumption in the later days of growth. The slow uptake of reducing sugars was probably due to two events; consumption of reducing sugar by the fungus for its growth and metabolic need and, production of reducing sugar by the enzymatic digestion of complex soy bean carbohydrates (Figure 1).

Table 1 - Characteristics of primers used in Real-time PCR

| Amplicon size | TM | %GC | Primer sequence | Primer | gene | No. |
|---------------|------|------|-----------------------|---------|-------------|-----|
| 200 bp | 52 | 63.2 | GCACTGGACCTGGTACCTC | forward | $\Delta 6$ | 1 |
| | 55 | 55 | GACCCGTGATGATCTGCTTG | Reverse | | |
| 224 bp | 53 | 61.3 | GCTCTTGTTCCTGGCTGC | forward | $\Delta 12$ | 2 |
| | 56 | 60 | CGAGTGCGAGATTCTCCAGG | Reverse | | |
| 228 bp | 54 | 47.6 | GACGACCTCAACAAGAGCAAA | forward | $\Delta 9$ | 3 |
| | 56 | 55 | CGGTCATCAAAGGGTCCATC | Reverse | | |
| 221 bp | 56 | 61.1 | GTTTGGGGCTGCAGATGC | forward | $\Delta 5$ | 4 |
| | 53 | 55 | CAAAGAGCTGCGCGTAGTAG | Reverse | | |
| 234 bp | 56 | 57.9 | CCCAAGGTCGGCAAAGTCT | forward | MALCE1 | 5 |
| | 55.5 | 55 | GCGTTGTTCACAAGCTCTG | Reverse | | |
| 221 bp | 55.7 | 47.6 | CCTTAAGAAGAACAACCGCCA | forward | GLELO | 6 |
| | 55.4 | 55 | GCGAACGCGTGATGTAGAAC | Reverse | | |
| 230 bp | 53 | 52.4 | CGACAACATGACTGGTACTGC | forward | Malic | 7 |
| | 57 | 52.4 | GCAGGGTTTCCTCAAGCTGAT | Reverse | | |
| 200 bp | 54 | 68.8 | CGAGTTGCGTGTCCGCC | forward | Actin | 8 |
| | 52 | 55.6 | TGGGAACAGTGTGGGTGA | Reverse | | |

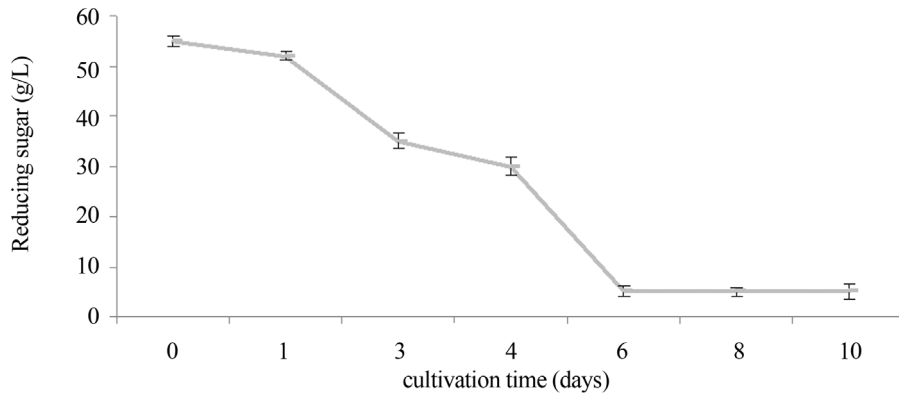


Figure 1 - Time courses of reducing sugar consumption by *Mortierella alpina* CBS 754.68. The vertical bars represent S.E. of the means of three replications.

The protein source did not deplete but remained in the medium until day 6. Dynamic analysis showed that the residual glucose was low but the nitrogen sources did not deplete. As soybean is a complex medium containing insoluble protein, these constituents presumably remained until day 6 in the medium (Figure 2).

Time courses of lipid content in biomass were measured (Figures 3). According to these results, the lipid con-

tent reached its peak at day 6. Lipid accumulation increased as reducing sugar significantly decreased from day 4 to day 6 (Figures 1 and 3). Comparison between protein consumption rates and those of lipid production implies that lipid accumulation increased in parallel with reduction in reducing sugar contents (Figures 1, 2 and 3).

Dry biomass yield of *Mortierella alpina* CBS 754.68 is shown in Figure 4, indicates a substantial increased in the

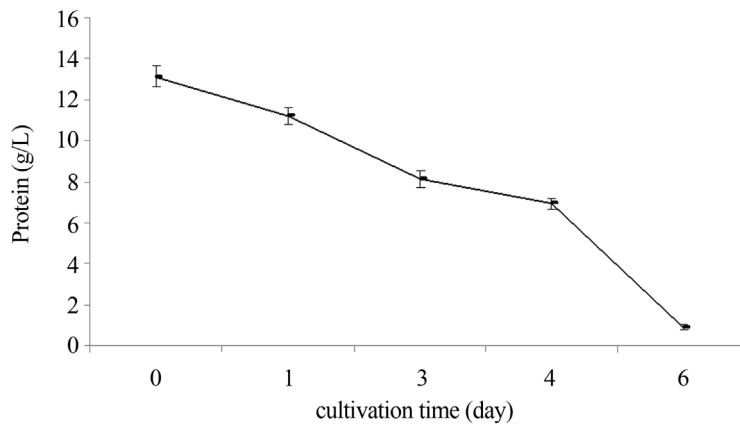


Figure 2 - Time courses of protein consumption by *Mortierella alpina*. The vertical bars represent S.E. of the means of three replications.

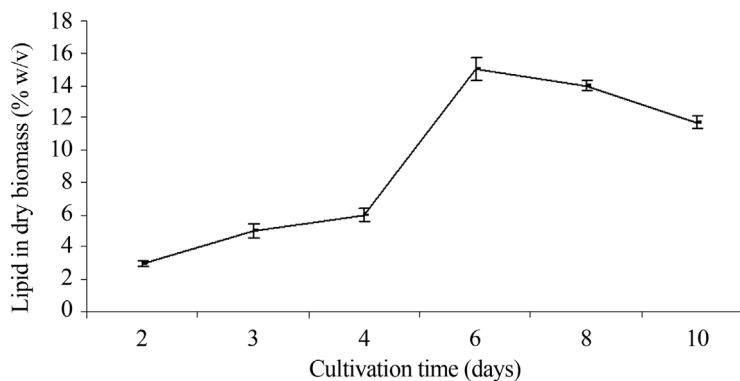


Figure 3 - Time courses of lipid production by *Mortierella alpina*. The vertical bars represent S.E. of the means of three replications.

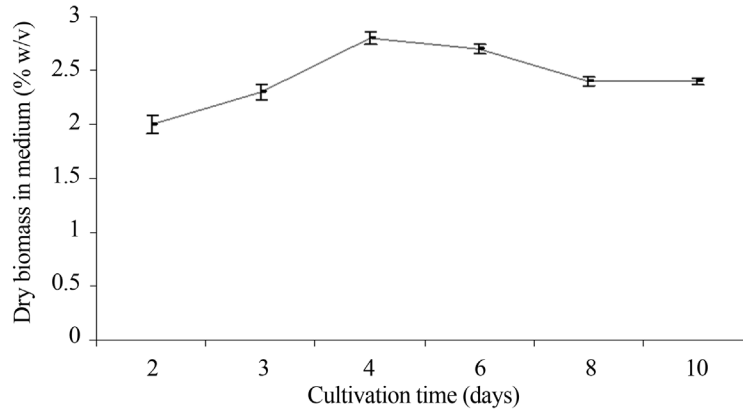


Figure 4 - Time courses of dry biomass production by *Mortierella alpina*. The vertical bars represent S.E. of the means of three replications.

early days of growth until day 2. Such increase may be due to the consumption and digestion of insoluble protein and complex carbohydrates present in soybean. However, the rate of yield production reduced after day 4 probably due to depletion of these substrates. Under these conditions, lipid accumulation increased substantially concomitant with the increased utilization of readily-digestible substrates such as soluble protein and monosaccharides (Figures 1 and 3).

It should be noted that while the biomass dry weight reached a plateau at the end of fermentation, the lipid amount decreased substantially (Figures 3 and 4). This suggests that lipids are the best macromolecules that store chemical energy and this is probably why they are deposited in *Mortierella* and many other oleaginous fungi (Weete, 1981). On the other hand, the stationary phase itself is an excellent phase for synthesis of fatty acids especially ARA (Jin *et al.*, 2007).

As shown in Figure 5, a substantial increase of ARA content in lipids occurred after the lipids decreased in biomass at the end of fermentation (Figures 3 and 5). The lipid content decreased from 15% at day 6 to 11.7% at day 10 and ARA content increased from 34% to 55%. These re-

sults are in agreement with the findings reported by (Zhu *et al.*, 2006; Eroshin *et al.*, 2002) demonstrating a great increase of ARA content in lipids after glucose exhaustion and lipid yield decrease. Also these results are in full accordance with other organisms. For instance, Funtikova and Mysyakina (1997) reported that gamma linolenic acid content in *Mortierella spinosa* becomes relatively low during lipid accumulation and increases as the lipids degrade.

The results presented in our study demonstrated that *Mortierella alpina* CBS 754.98 has high phenolic content and thus could be potential rich sources of natural antioxidants (Figures 6). The phenolic compounds appear to have an important role in reducing lipid oxidation, associated with their antioxidant activity (Samadlouy *et al.*, 2008). However, the phenolic content had no association with lipid and ARA accumulation and was only related to cell growth (Figures 4 and 6). The maximum phenolic yield was obtained after 4 days of cultivation (73 mg gallic acid equivalents/ 100 g) which is a decreasing trend, thereafter compared to other edible mushroom species such as beefsteak fungus (Ribeiro *et al.*, 2007). *Mortierella alpina* CBS 754.98 apparently contains more phenolic compounds,

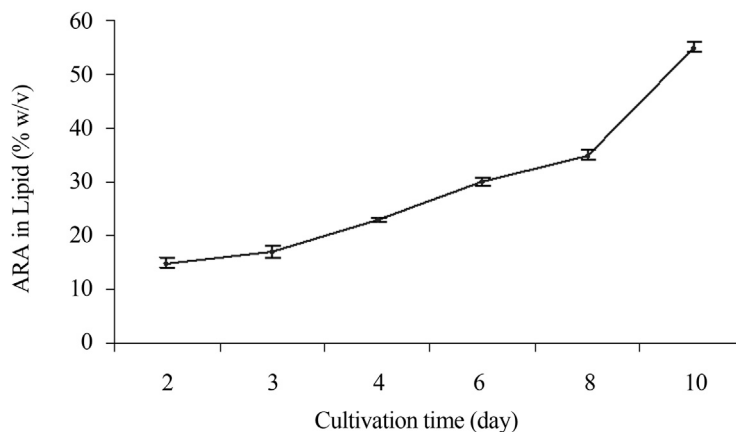


Figure 5 - Time courses of ARA production by *Mortierella alpina*. The vertical bars represent S.E. of the means of three replications.

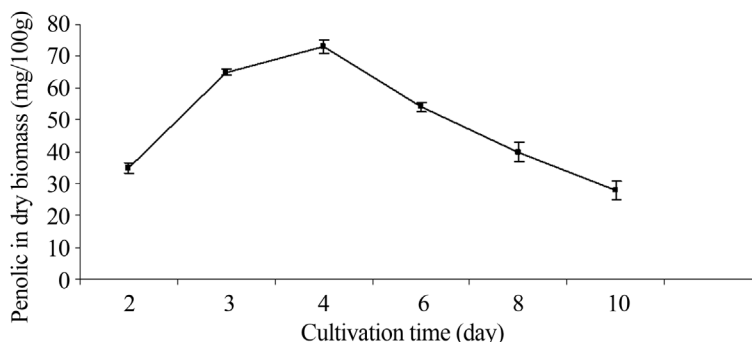


Figure 6 - Time courses of phenolic production by *Mortierella alpina*. The vertical bars represent S.E. of the means of three replications.

which highlights the importance of this fungus as a rich source for these compounds.

Fakas *et al.* (2008) reported that hydrogen peroxide content in the lipid of the oleaginous fungus increased from the exponential step to the stationary phase. It is conceivable from our results that this increased level of hydrogen peroxide may be related to the overall reduction of intracellular phenolic content and increased desaturation of fatty acids levels at the end of fermentation.

The expression levels of desaturase and elongase encoding genes

At days 2 and 4, in which dry biomass increased, the ARA content slowly increased in parallel with reduced expression of $\Delta 12$ encoding gene at day 2 and GLELO encoding gene at days 2 and 4. In fact, at the same day of cultivation, intermediate fatty acids contents (such as stearic acid, oleic acid and linoleic acid) increased substantially concomitant with decreases in the expression level of the two above mentioned genes. According to Gray *et al.* (2002), fatty acids in cell membranes are usually 18 carbon fatty acids.

We found that intermediate unsaturated fatty acids critical for cell growth (such as stearic acid, oleic acid, linoleic acid) were increased. Therefore, the reduced levels

of $\Delta 12$ and GLELO encoding genes expression in the growth phase could be a reflection of their role in the protection of *Mortierella alpina* CBS 754.68 through membrane fluidity.

The expression levels of the desaturase and elongase encoding genes were the lowest when carbon and protein contents reduced to 5 and 1 g/L at day 6, respectively. These genes were highly expressed at day 8 when an accelerated reduction of lipid content in dry biomass was observed (Figures 7 and 8). It is believed that the shift in the energy source from glucose to lipid could be the main factor leading to the increased rates of desaturase and GLELO encoding genes expression at day 8. The analysis of expression of desaturase and GLELO encoding genes indicated that the elongase converting 18:3(*n*-6) to 20:3(*n*-6) is a rate-limiting enzyme in the ARA biosynthesis in the early growth especially until day 4. However, at the end of growth when the energy source shifted to lipids, the GLELO encoding gene no longer acted as the rate limiting gene in the biosynthesis of ARA.

The expression levels of malic enzyme encoding gene

The expression level of the malic enzyme encoding gene was the highest at days 2 and 4 (Figure 9), which was

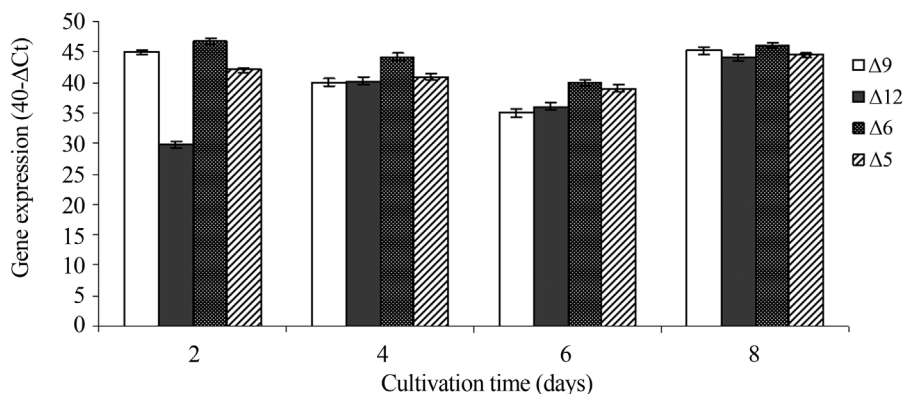


Figure 7 - Gene expression ($40-\Delta Ct$) of desaturase encoding gene by Real-time PCR analysis. Real-time PCR amplification of actin was used to normalize expression of the genes under identical conditions. The vertical bars represent S.E. of the means of three replications.

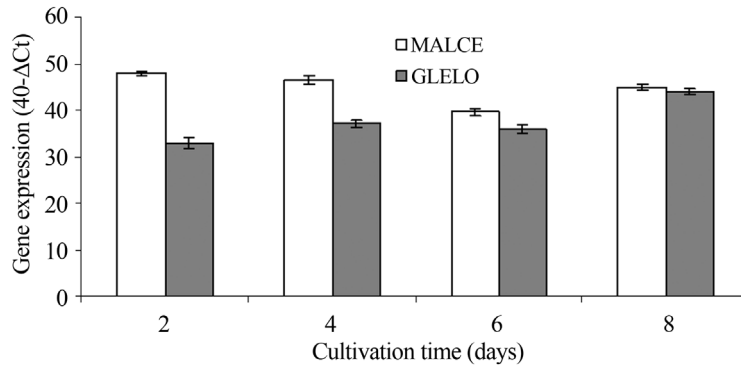


Figure 8 - Gene expression (40-ΔCt) of elongase encoding genes by Real-time PCR analysis. Real-time PCR amplification of actin was used to normalize expression of the genes under identical conditions. The vertical bars represent S.E. of the means of three replications.

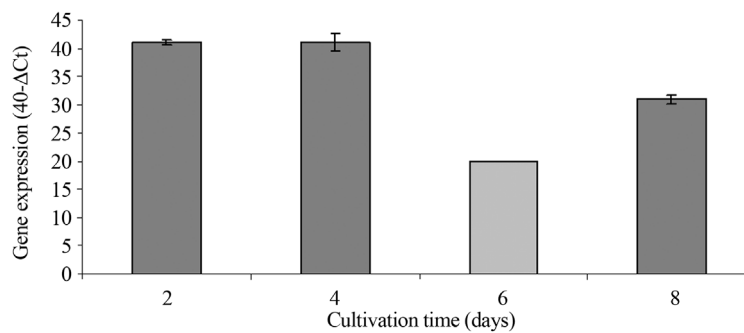


Figure 9 - Gene expression (40-ΔCt) of malic enzyme encoding gene by Real-time PCR analysis. Real-time PCR amplification of actin was used to normalize expression of the gene under identical conditions. The vertical bars represent S.E. of the means of three replications.

in parallel with increasing in the lipid and biomass contents in the early days of cultivation. This might imply that malic enzyme encoding gene is closely bound to lipid and biomass biosynthesis. The results were similar to those obtained by Wynn *et al.*, 1999 who reported malic enzyme which plays an important role in the provision of NADPH for lipid biosynthesis in *Mortierella alpina*. Under these conditions other fatty acids were slowly converted to ARA. Thus, the increase in lipid accumulation may have the most profound effect on lipid composition. These results are in agreement with studies demonstrating that PUFA concentration decreases along with lipid content increase (Hansson and Dostalek, 1988; Hansson *et al.*, 1989; Chen and Chang, 1996; Gema *et al.*, 2002; Papanikolaou *et al.*, 2004; Fakas *et al.*, 2009). Reducing sugar and protein content reduced to 5 and 1 g/L at day 6, respectively, resulting in a low basal level of expression of the malic enzyme encoding gene (Ct = 40) followed by inhibition of lipid production (Figures 1, 3 and 9). The results were similar to those obtained by Wynn and Ratledge (2000) who reported a relatively increased activity for the malic enzyme following *Mortierella* strain growth on glucose. The expression of the mentioned gene increased at day 8 and its 40-ΔCt reached 31 (Figure 9). This increase in the expression of the malic enzyme encoding gene could be due to its important role in providing NADPH for ARA production (Figures 5 and 9).

The results are in accordance with those obtained by Kendrick and Ratledge (1992) who reported that the malic enzyme in oleaginous species acts as a provider of NADPH for fatty acid desaturation and even elongation.

There are two assumptions for ARA accumulation in oleaginous microorganisms in batch culture. According to Eroshin *et al.* (2002) ARA content in lipids increases as a result of conversion of other fatty acids. In this study, the increase in the ARA content in the lipids at the end of fermentation could be related to the overall reduction of cellular metabolic activity. This down-regulation would then restrict carbon flow through the metabolic pathway reducing the expression of the malic enzyme encoding gene and production of reducing powers (*e.g.* NADH, NADPH). According to Georgiou *et al.* (2006) the diminished concentration of cell reductants would increase the intracellular oxygen concentrations. Therefore, it can be concluded that the elevated oxygen concentrations observed in this study resulted in the increased expression of GLELO and desaturase encoding genes which converts other fatty acids to ARA.

Conclusion

The results of this study showed that the expression of our target genes depends on culture conditions. It can be concluded that the ARA accumulation increases in parallel

with reduced expression of the malic enzyme encoding gene that coincided with reduced levels of lipid in dry biomass.

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