

Full Length Research Paper

Improving the optimum yield and growth of *Chlamydomonas reinhardtii* CC125 and CW15 using various carbon sources and growth regimes

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Chlamydomonas reinhardtii CC125 (wild type) and CW15 (cell wall mutants) were feed up on solid and liquid Tris phosphate (TP) media with various concentrations of acetate, glycerol(10-100 mM) or methanol (0.01-718 mM) and cultivated under phototrophic, mixotrophic and heterotrophic conditions. Use of 10 and 35 mM acetate and 10 and 50 mM glycerol under constant $38 \mu\text{E m}^{-2} \text{s}^{-1}$ light illumination (mixotrophically) was the optimum condition for both strains to have a higher biomass and growth compared to other carbon sources and concentrations. Both strains had a quicker growth rate in just 35 mM of acetate and 10 mM glycerol although feed of algal cells on 35 mM acetate produced more and quicker biomass. In use of 10 mM acetate in micro plate and tissue culture flasks, CW15 had a maximum growth rate of 5.3×10^4 and 1.3×10^4 cells/hour; while on use of 35 mM acetate, the growth rate was 8.8×10^4 (micro plate) and 4.0×10^4 cells/hour (tissue culture flasks). Wild type had a maximum 2.7×10^4 (micro plate) and 4×10^3 (tissue culture) cells/hour in use of 10 mM acetate. In feed of CC125 with 35 mM acetate, growth rate correspondingly for micro plate and tissue culture flasks was 2.5×10^4 and 2.6×10^4 cells/ hour. Among the two strains, CW15 with specific growth rate of 8.8×10^4 cells/hour (in micro plates) and 4.0×10^4 cells/hour (in tissue culture flasks) on 35 mM acetate also grew quicker than CC125. Susceptibility to bacterial contamination was checked on both strains and we also found that, just as the absence of a cell wall in CW15 accelerated the growth, it also appeared to increase the chance of contamination by about twofold compared to the wild type but this can be minimized by the use of antibiotics in the growth media.

Key words: Heterotrophic growth, mixotrophic growth, acetate, glycerol methanol *Chlamydomonas*, CC125, CW15.

INTRODUCTION

Reducing the cost of bio-products processes from its earlier stage to the final one is a vital factor that has

been carried out throughout the world. Although discovery of some bacteria such as *Escherichia coli*

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reduces bio-process applications for many decades, it is time to substitute an alternative organism in which to mount the yield of biomass with minimal inputs and costs. Microalgae are a kind of organism that seems to have this functionality in addition to other advantages ranging from (1) their accessibility from fresh water and soil, (2) photosynthetic ability, and, (3) well-annotated genomes such as *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Volvox carteri*, *Chlorokybus atmophyticus*, and *Dunaliella salina* to just name few.

The green algae *C. reinhardtii* among other micro algae specious has been utilized as a model species in many bio-applications (Roacha et al., 2013; Fischer et al. 2006; Ledford et al., 2007; Peers et al., 2009). Generally, it can grow phototrophically and this means it uses incident light and CO₂ and converts it to sugar and O₂. Some strains of *C. reinhardtii* have the capability to grow mixotrophically and/ or heterotrophically (Brennan and Owende, 2010; Dragone et al., 2010). Despite heterotrophic feed of microalgae that let algal cell consume solely the external organic carbon source, microalgae which can grow under mixotrophic growth condition can form synthesized carbon source and has both photosynthetic and heterotrophic characteristic. However, in mixotrophic growth regime microalgae consume simultaneously light and external organic carbon source for its growth (Dragone et al., 2010).

Production of microalgae in large-scale is somehow considered as a key parameter for industrialization and commercialization of microalgae and has been studied for decades (Becker, 1994). The ease of its, well annotated genomes and cells behavioral make it as a remarkable laboratory tool to be used in wide range variety of either practical or potential products ranging from lipid, enzymes, isoprenoids, polysaccharides cultivation as well as the production of biofuels as a green and substitute energy source to traditional fossil fuels (Brennan and Owende, 2010).

The achievement of above mentioned product (enzymes, biofuel, lipid and polysaccharides cultivation), mostly was occurred by cultivating the microalgae on varied mineral media, organic substrates, and synthetic or real wastewaters (Pulz, 2001; De-Bashan et al., 2002; De-Bashan et al., 2004; Pulz, 2004; Lebeau et al., 2006; Harun et al., 2010).

Today, growth of micro algae under phototrophic condition is the most common method compared to mixotrophic and heterotrophic conditions. However, the most common one does not necessarily mean the best one, as literatures has shown that the use of both light and external carbon source in mixotrophic condition not only causes a higher yield but also reduces the cost of cultivation compared to phototrophically growth condition (Bhatnagar et al., 2011; Cerón García et al., 2005) and this is commercially quite vital (Abad and Turon, 2012; Liang et al., 2009).

Also, Yu et al. (2009) (Shi et al., 1999) has shown that

growing algae via heterotrophic and mixotrophic conditions can overcome such disadvantages of phototrophic growth (low cell densities and longtime cultivation) and the culture conditions for optimum growth and yield rate have not been suggested therefore a think of suitable organic carbon among several carbon sources can be challenging and crucial. To our knowledge, the most common used organic carbon sources for heterotrophic and mixotrophic algal growth are glucose, acetate, sucrose, lactate, lactose, ethanol and glycerol (Shi et al., 1999; Chen and Johns, 1996; Octavio et al., 2010; Zhang et al., 1999).

Despite the importance of right carbon sources and right growth conditions, several factors such as algal strains with various characteristic and behavior, pH, oxygen level, association with or without bacteria or fungi, temperature and introduction of adequate light intensity (Ras et al., 2013; Spreitzer et al., 1998; Falk et al., 2006) to permit a faster algae growth are key objectives that may alter the biomass yielding (Mandalam and Palsson, 1998; Yang et al., 2000; Suh and Lee, 2003). Here our aim is to examine the wild type (CC125) and the cell wall mutants (CW15) of *C. reinhardtii* algae grown on different types of carbon sources in particular (acetate, glycerol and methanol) at various concentration in both dark and light conditions to suggest an optimal culture condition for achieving a higher growth yield and biomass.

Acetate (or acetic acid) is one of well-known carbon sources that its use has been generally accepted for variety of both microbial species and microalgae (Droop, 1974; Moon et al., 2013). Generally, acetate can be metabolized through two routes when it has been carried by coenzyme A. The first pathway will occur from glyoxylate cycle in order to produce malat and the second pathway that can provide the energy (ATP and NADH) and carbon skeletons through the tricarboxylic acid (TCA) to citrate cycle. Solely, those micro algae species that has the first metabolic pathway can grow as the two crucial enzymes isocitrate lyase and malate synthetase (which themselves are necessary for operating the glyoxylate cycle) later will be stimulated in algal cells when they are in acetate media (Neilson and Lewin, 1974; Boyle and Morgan, 2009).

Albeit solely some types of micro algae such as *Phaeodactylum tricornutum*, *Nannochloropsis* sp., *Rhodomonas reticulate*, and *Cyclotella cryptica* can consume glycerol as a carbon source, optimizing the use of glycerol for a faster growth and higher yield in mixotrophic condition has been done for decades (Choi et al., 2011; Cero'n Garcí'a et al., 2000). It is a by-product of biofuel production and a good source for preservation of microorganism including microalgae at low temperatures (Moon et al., 2013). Despite acetate that in high concentration will caused cells degradation due to its acidity and toxicity characteristic, glycerol

has not have any toxicity effect on cells even at higher concentration (Kaplan et al., 1986) and so far several efforts have been done to utilize this non-toxic organic carbon source in microalgae growth.

Methanol is the most basic form of alcohol and in its use in biofuel processing it plays a key role to convert triglycerides (TGA) into biodiesel through a mechanism called transesterification. Introducing the methanol into algal system for instance (0.01-718 mM) compared to other carbon sources (glucose, acetate glycerol) has an advantage as it can sterilize the media and therefore minimize bacteria and fungi contamination in open ponds (Choi et al., 2011). Methanol frequently has been used in commercial purposes due to its low cost and the fact that is a biofuel by-product. In a study by Choi et al. (2011), it was shown that growth of *Chlorella* in 1% (v/v) methanol can increase the lipid yield and growth rate (Moon et al., 2013). However, the use of methanol either mixotrophically or heterotrophically has not been reported as an organic carbon source has been reported a level of flocculation in *C. reinhardtii* algal cells (cite methanol algae article). By consideration of merit and demerit points of each mentioned organic carbon source in this study we will conclude the most efficient carbon source for faster growth and yield.

MATERIALS AND METHODS

Solid cultures

In a 500 ml bottle, Tris-Phosphate in absence of acetic acid medium (TP) (Gorman and Levine, 1965) was mixed with 2% Difco agar and autoclaved for 15 minute at 121°C. Then acetate and glycerol with various concentrations (10, 20, 35, 50 and 100 mM) were individually added in a separate 500 ml TP + 2% agar media and they also autoclaved for 15 min at 121°C.

Methanol (0.01, 25, 123, 245 and 718 mM) was added to the sterilized TP media. Each of the above prepared media (TP, Acetate, Glycerol and Methanol) was poured in 8 Petri dishes (two for each strain one for light and one for dark condition). Each axenic algal strain which was initially grown on TAP media + 2% agar and was taken from Professor Colin Robinson's lab, inoculated and streaked into the above solid prepared TP, TP-Acetate, TP-Glycerol, TP-Methanol media and was grown for 7 days in light with intensity of $38 \mu\text{E m}^{-2} \text{s}^{-1}$ and dark condition.

Liquid culture

Micro-plate

Into two 24 wells micro plates (Cellstar company) 1.5 ml of TP (as phototrophic control), Acetate or Glycerol (10, 20, 35, 50 and 100mM) and (0.01, 25, 123, 245 and 718 mM) of methanol independently was pipetted twice. Algal cells were inoculated and mixed in a separate 1.5 ml Eppendorf tube containing sterilized TP media and then 50 μL of each strain individually was pipetted into each well of the micro- plate with specific mentioned acetate, glycerol and methanol concentration. For mixotrophic growth one of the two microplates was put under light intensity of with $38 \mu\text{E m}^{-2} \text{s}^{-1}$ and the other one covered with aluminum foil for heterotrophic purposes. Both micro-plates then were put in the

algae room on a separate *Panasonic* double orbital shaker with speed of 170 rpm for 7 days. Once cells were grown for one week, growth rate were visualized and only acetate and glycerol mixotrophically seemed to be utilized by algal cells with certain concentrations seemingly be used more efficiently, that is, 10 and 35mM of acetate and 10 and 50mM of glycerol. Consequently, each strain once more was grown at that efficient concentration of carbon sources in triplicate in a light intensity of $38 \mu\text{E m}^{-2} \text{s}^{-1}$ in both 24 well micro plate. Daily biomass rate was checked by a *Panasonic* micro plate reader in 600 nm wave length. Sample also was taken from each micro plate well and cells counted by *Neubaur* hemocytometer (Figure 5, part C and D, illustrate the callibration of cell numbers with absorption in 600nm).

Tissue culture flasks

To find out a possible role of different shaking speed in algal respiration system, the experiment was repeated at half the speed of 24 wells micro plate (70 rpm) but in tissue culture flasks. A set of three of Sigma-Aldrich 25 cm^3 tissue culture flasks independently was used for each concentration of chosen carbon source. Each set then separately filled with 25 ml of prepared TP with (10 and 35 mM) acetate and (10 and 50 mM) glycerol. In each flask, 100 μl algae cells from each strain was pipetted. Flasks were thereafter put on a 70 rpm *Panasonic* double orbital shaker for 7 days. Cell counting, optical density at 600 nm (Figure 5, part A and B, display the callibration of cell numbers with absorption in 600nm) and chlorophyll measurement was measured daily.

Chlorophyll extraction and measurement

Chlorophyll extraction from tissue culture flasks

One milliliter from each tissue culture flasks containing algal cells was pipetted into individual 1.5 ml centrifuge Eppendorfs and centrifuged for 5 min at 6000 rpm. Supernatant was removed and algal cells vortexed with 1 ml 95% ethanol (v/v) for about 45 s. Eppendorfs were again centrifuged for 4 min at 13,000 rpm. The remaining supernatant containing extracted algal chlorophyll was transferred in 1 ml plastic cuvette. The spectrophotometer was blanked against 95% ethanol before each absorption at 649, 665 and 750 nm.

Chlorophyll a counting

The rate of produced chlorophyll a calculated via the below equation (Bergmann and Peters, 1980):

$$\text{Chl a } (\mu\text{g/L}) = \frac{(13.7(A_{665} - A_{750}) - 5.76(A_{649} - A_{750})) V}{v * l}$$

Where, V is the volume of extract in ml; V is the volume of sample filtered in; l is the length of cuvette in cm.

Algal susceptibility to bacterial contamination

Algae strains from solid TAP media were inoculated and mixed with 1.5 ml fresh liquid TP media and vortexed for 2 min. Algae cells were diluted 100 times and cell density was measured by counting the cells in a hemocytometer (CW15 and CC125 respectively had 1.25×10^6 cells/ml and 5×10^5 cells/ml). Our aim was

to observe 3000 bacterial colonies in use of 1ml algal culture meaning in 0.1 ml of algae culture we would expect to see 300 bacteria colonies. Samples were diluted appropriately to give 300 colonies per plate. Two percent agar from *Difco* Company was poured individually in a 100 ml of TP, TAP and TSB (Tryptone-Soya Broth) and autoclaved as before.

The autoclaved media independently poured in 4 individual sterilized petri dishes. 100 μ l diluted sample were pipetted into 4 individual solid TP, TAP and TSB petri dishes and distributed perfectly in all petri dishes. While TSB petri dishes were kept in a 30°C incubator, TP and TAP Petri dishes were kept in 27°C algae growth room with 38 μ E m⁻² s⁻¹ light intensity.

Physical morphology

Both algal strain which grown without additional of any carbon source in solid and liquid TP, TAP were visualized under the *Leica DMR* (Digital Module R) microscope with 1000 magnification and compared to the grown algae cells in various concentration of acetate, glycerol (10, 20, 35, 50 and 100 mM).

RESULTS AND DISCUSSION

Physical morphology

Visualization of both strains under light microscope with 1000X magnification showed the existence of cell wall and flagella respectively in CC125 and its absence in CW15. Despite having an approximate same size (50 μ m) and circular shape of both strains, cells in CC125 mostly joined together as a colony in a capsulated environment by their cell wall while the CW15 cells remained separate from each other.

Moreover, further microscopy of the grown CC125 algal cells either mixotrophically or heterotrophically with different concentrations of acetate (10, 20, 35, 50 and 100 mM) indicated some valuable results. For instance, in dark growth conditions using 50 and 100 mM acetate the color of existed chlorophyll in CC125's cells mostly was bright yellow rather than green (Figure 7, part C and D clearly show the differentiate color of chlorophyll).

The reason that chlorophyll looks green is that it absorbs other colors existed in light (red and blue) except green. Also, in low light intensity more rate of chlorophyll will expect to produce. And the more chlorophyll means more green color (Lange et al., 1981). Therefore, this may suggest an existence of a deficient in CC125 chloroplast pigments (chlorophyll a, b or xanthophyll) in which the absence of light and high rate of concentration (50 and 100 mM) inhibits algal cells to either absorb the light or consume high concentrated acetate for growth. Another possibility which also was seen in 24 wells micro plate can be cell degradation and death due to the high toxicity of 50 and 100 mM acetate. Figure 7A shows cell degradation and death while part B display steady cell aggregation). We also found that in heterotrophic use of 50 and 100 mM acetate most of CW15 algal cells lost their circular shapes and instead formed some

unstructured shapes. In Figure 1, a noticeable difference in shape of CW15 in use of low acetate concentration (B) and high acetate concentration (D) was observed.

The optimum condition and concentration for acetate in solid and media culture

From the data gathered after a period of seven days we found that mixotrophic growth of both strains was much higher than growing under either phototrophic or heterotrophic conditions. Moreover, mixotrophic growth of algal cells on acetate, glycerol and methanol revealed that acetate was the carbon source that was consumed better compared to the others. Our experiment also showed that the optimum concentration of acetate for improving the yield was 35 mM. Figure 2A indicates a higher mixotrophic growth compared with other carbon sources (C&E) and higher concentration of acetate (100 mM) caused cell death and degradation specifically in CC125 which might be due to the toxic effect of 100 mM acetate on algal cells. A rate of flocculation also was seen on CW15 when a range of methanol concentration (0.01, 25, 123, 245 and 718 mM) was used mixotrophically (Scholz et al., 2011).

The maximum growth rate of CW15 grown on micro plates in feed of 10 and 35 mM acetate correspondingly was 5.3×10^4 and 8.8×10^4 cells/hour. The wild type (CC125) showed less growth rate, being 2.7×10^4 (10 mM) and 2.5×10^4 (35 mM) cells/hour. Figure 3A and B show algal cell growth in 10 and 35 mM acetate.

Results given from tissue culture flask also has shown that regardless of shaking speed and its possible effect on algal respiration system, CW15 grew quicker on 35 mM acetate with maximum rate of 4.0×10^4 cells/hour than CC125 with maximum rate of 2.6×10^4 cells/hour. Figure 4C and D reflect the faster growth of CW15 on 35 mM acetate feed. The CW15 also had a faster chlorophyll production rate of $0.046 \mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ compared to CC125 with a rate of $0.008 \mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ (Figure 6).

We also found that in mixotrophical use of 35 mM acetate both strains produced a higher biomass than other concentrations and conditions. The maximum rate of extracted chlorophyll a, for instance, in 35 mM acetate was $17.89 \mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ (CC125) and $12.516 \mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ (CW15) which was noticeably higher than the phototrophic yield with rates of $0.467 \mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ (CC125) and $0.103 \mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ (CW15). Utilization of 10 mM acetate however produced lower chlorophyll a than 35 mM acetate but higher than phototrophic growth as CC125 had $10.95 \mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ and CW15 had solely $4.758 \mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$.

The optimum condition and concentration for glycerol

In mixotrophic utilization of 10 and 50 mM glycerol

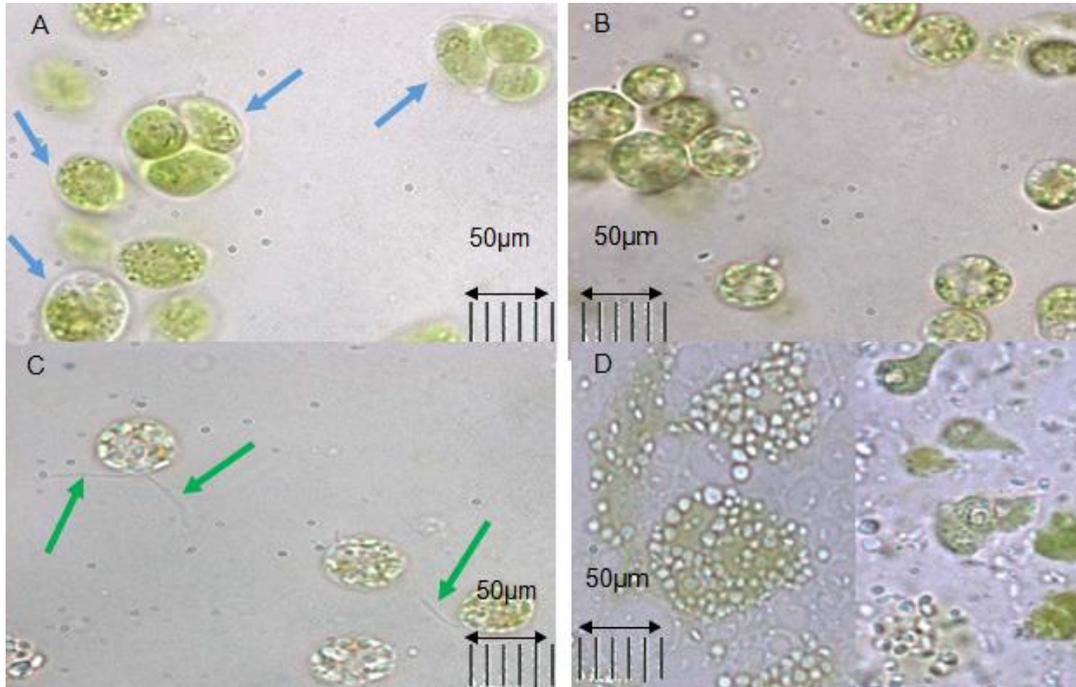


Figure 1. Physical morphology of both strains in magnification of 1000x. The blue and green arrows in A and C respectively show the existence of cell wall and flagella in CC125. B shows the CW15 that lacks both cell wall and flagella. The pale color of algae in picture C is because of the use of 20mM acetate in dark condition which may suggest the defect in its chloroplast system in dark condition. Although the size of each cell in both strains approximately is the same, the wild type (CC125) usually makes a colony of three or more cells which are capsulated by its surrounded cell wall (Picture A). Picture D shows unstructured shape of CW15 in use of 50 mM acetate in heterotrophic condition acetate. The last green column in C can show the lowest cell growth due to high toxicity of acetate). The blue and green arrows in A and C respectively illustrate cell wall and flagella in CC125 while B shows the lack of them in CW15.

however the outcomes from micro plates and tissue culture flasks were considerably different. In micro plates the maximum growth rate of CW15 respectively for 10 and 50 mM glycerol was 5.2×10^4 and 7.5×10^4 cells/hour while in tissue culture flasks cells were grown better on 10mM than 50mM as 2.4×10^4 and 1.3×10^4 cells/hour respectively were generated

The CC125 we had a higher growth in 10 mM glycerol in both micro plates and tissue culture flasks. The maximum growth rate in micro plate and tissue culture flasks was 2.0×10^4 and 7.8×10^4 cells/hour respectively. In 50 mM glycerol media results were 1.2×10^4 cells/hour in micro plate and 2.8×10^4 cells/hour in tissue culture flasks. This is how the maximum phototrophic growth rate in CC125 and CW15 in two times replicate respectively was 3.4×10^4 and 5×10^3 cells/hour and 4.9×10^4 and 6.5×10^4 and 2.3×10^4 and 1.1×10^4 cells/hour (Figures 3 and 4.).

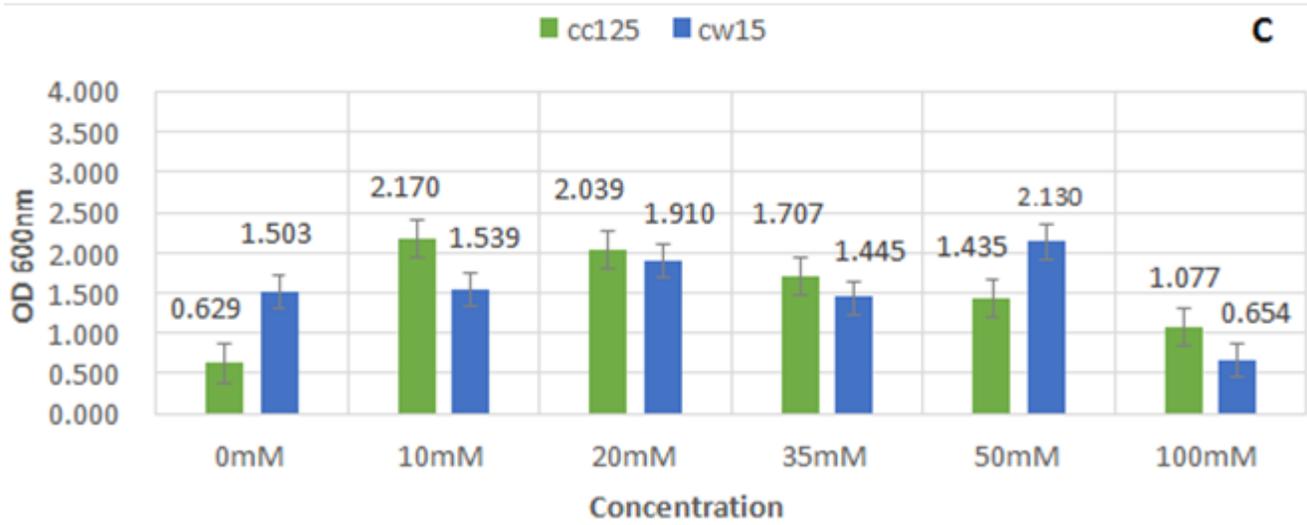
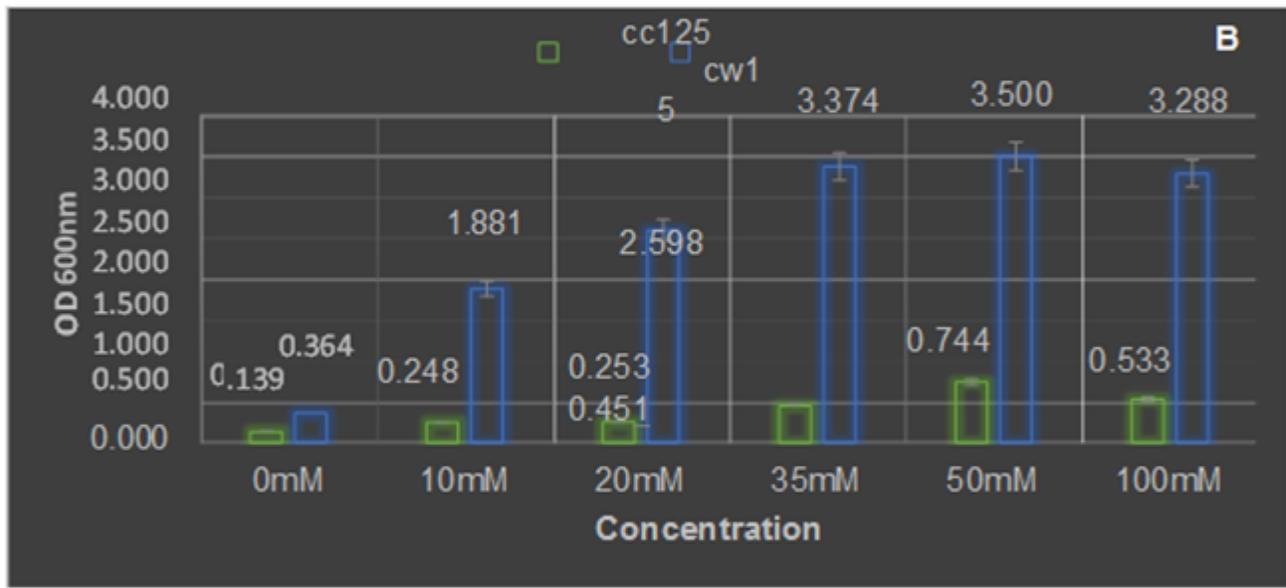
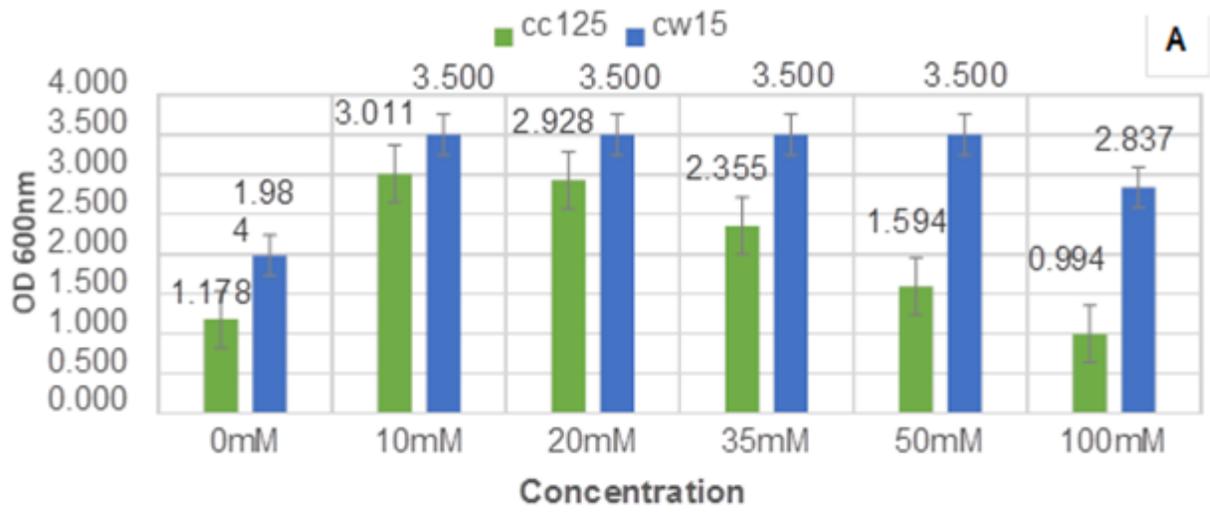
Extraction of chlorophyll from algal cells were grown on tissue culture flask also showed that CW15 and CC125 both had a faster chlorophyll production growth rate in 10 mM glycerol with corresponding rate of 0.037 (CW15) and 0.020 (CC125) $\mu\text{g.L}^{-1} \text{h}^{-1}$ than feed on 50 mM glycerol

with maximum production growth rate of 0.022(CW15) and 0.016 $\mu\text{g.L}^{-1} \text{h}^{-1}$ (CC125) (Figure 6C and D). However, the rate of produced biomass (chlorophyll a amount) in use of 50 mM glycerol was higher in CC125 than 10 mM (0.769 and 0.458 $\mu\text{g.L}^{-1} \text{h}^{-1}$, respectively). The cell wall mutants produced a same amount of chlorophyll a in use of 10 and 50mM glycerol (0.168 $\mu\text{g.L}^{-1} \text{h}^{-1}$). Likely to use of acetate, feed of algal cells on glycerol culture either in 10 or 50 mM concentration, showed a higher yield than phototrophic condition with rate of 0.467 $\mu\text{g.L}^{-1} \text{h}^{-1}$ (CC125) and 0.103 $\mu\text{g.L}^{-1} \text{h}^{-1}$ (CW15).

The data suggests that growing CC125 on small sized culture such as micro plate may not be industrial while the growth rate of phototrophic was higher than 10 and 50 mM glycerol however in bigger culture (tissue culture flasks) the use of just 10 mM glycerol can be quite interesting.

Economics matter in use of 10 or 50 mM glycerol

An important point in yield and growth of algae is economic matters in which to reduce the cost of



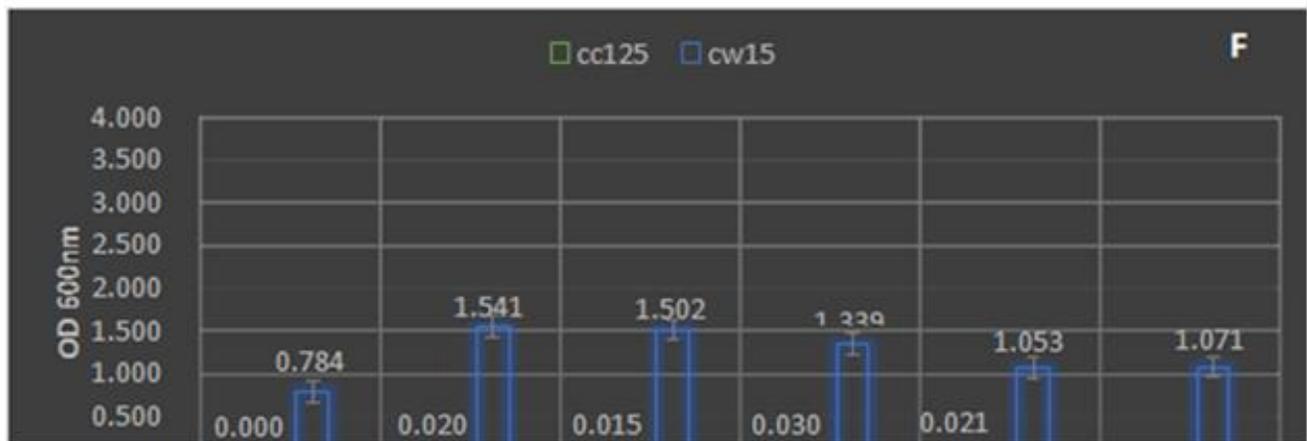
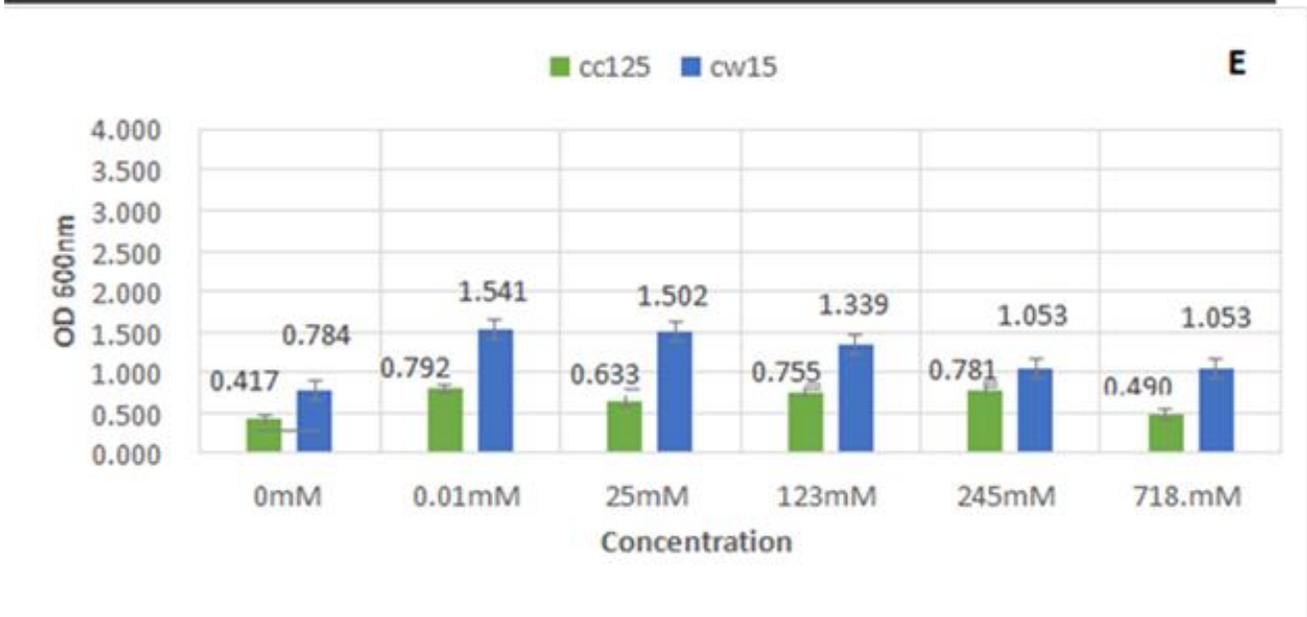
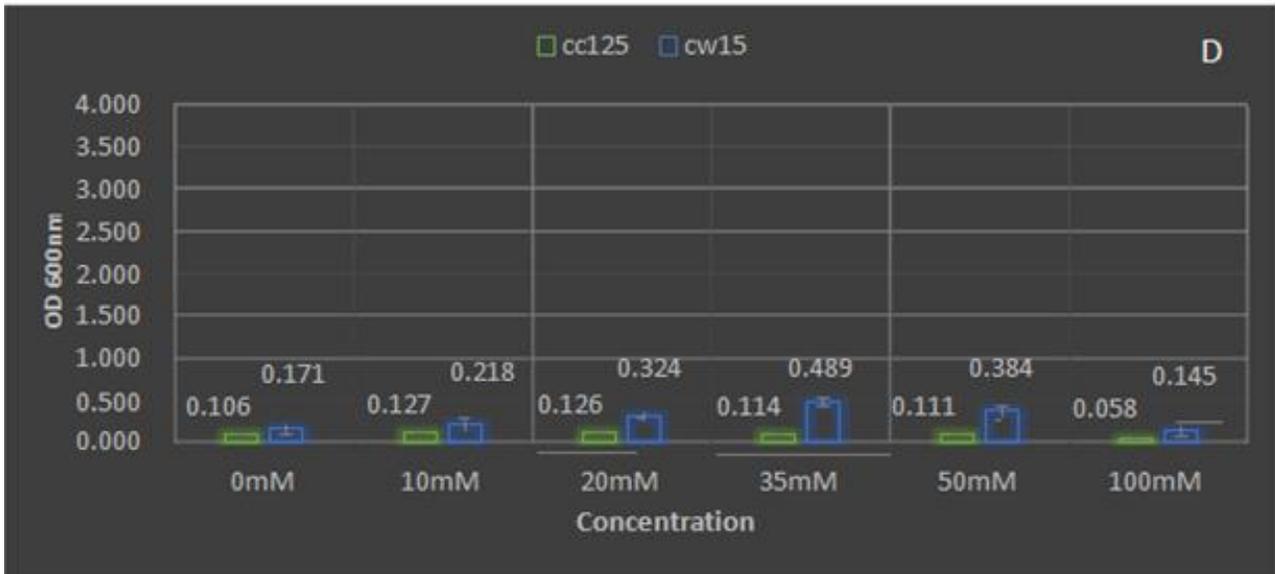
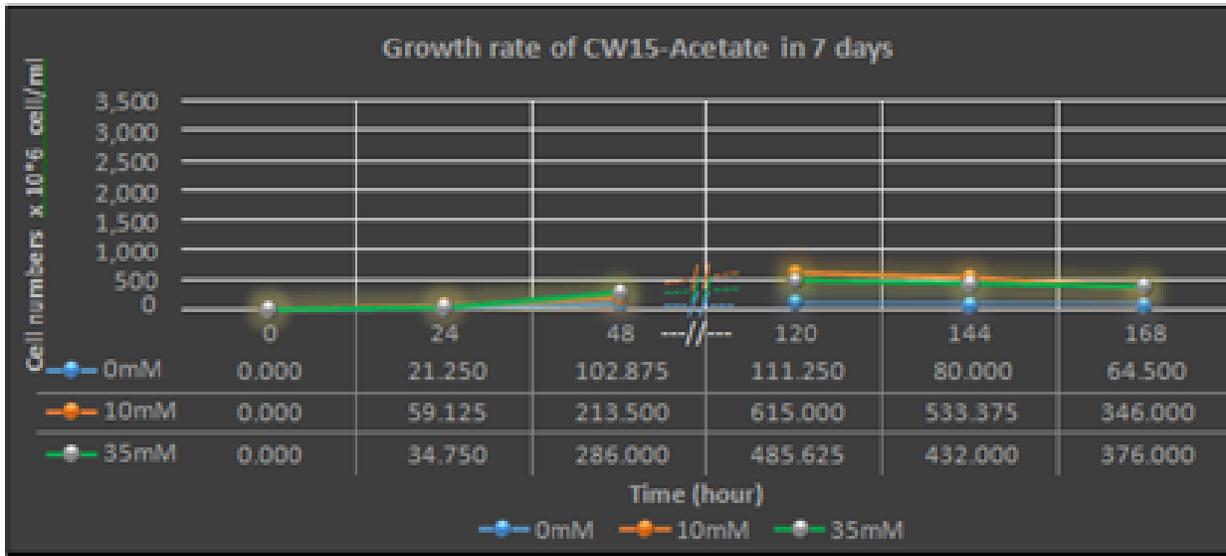
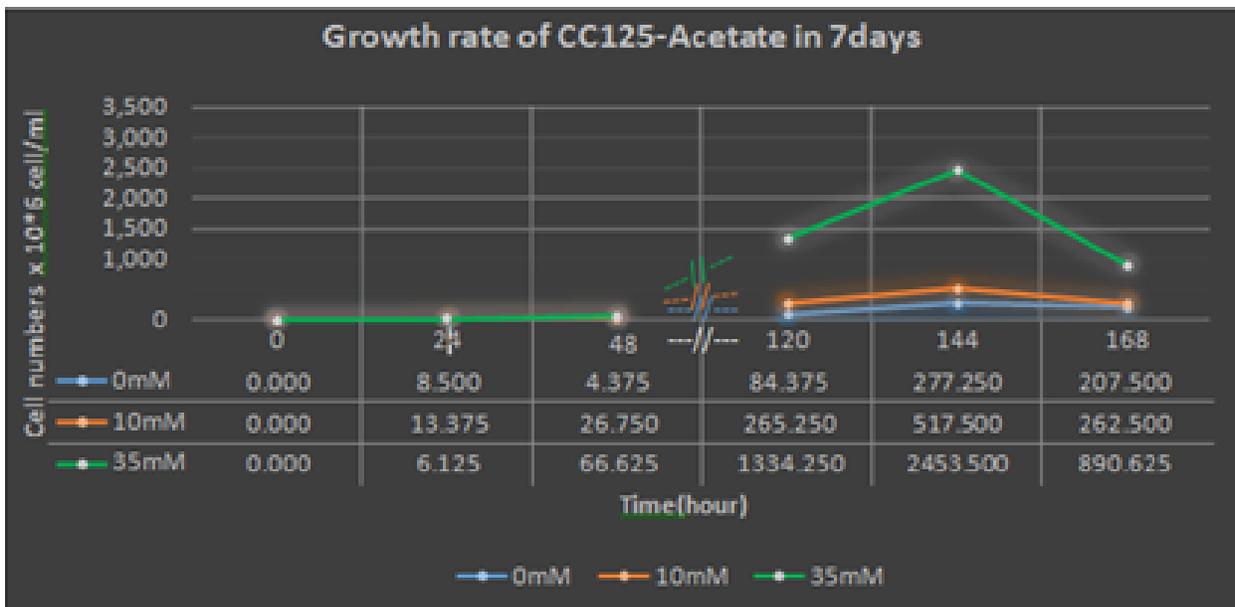


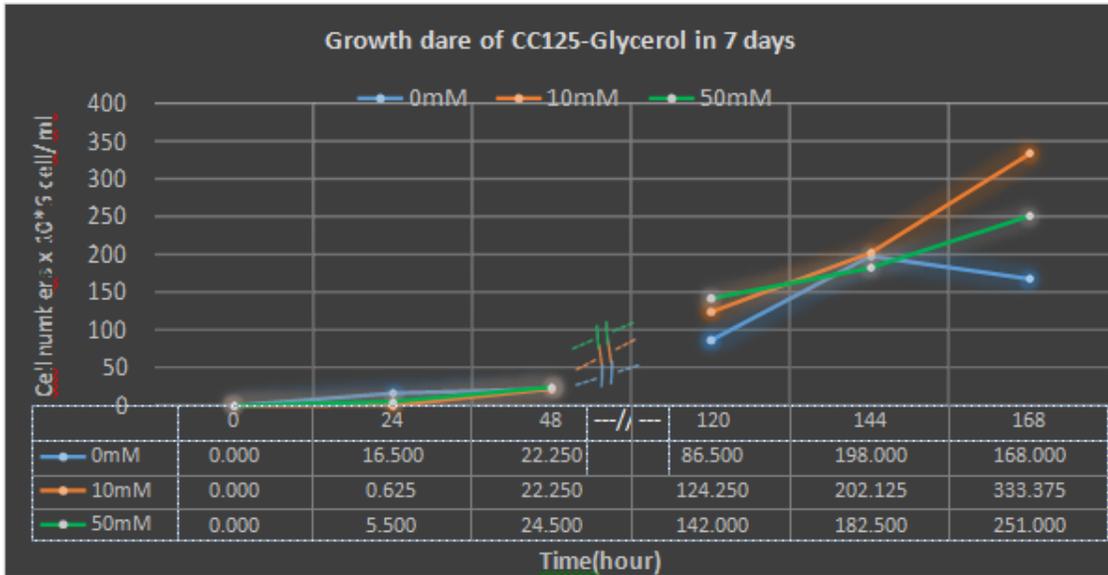
Figure 2. Various concentrations of different C-sources. Graphs illustrate the growth of microalgae (CC125 and CW15) in acetate (A-B), glycerol (C-D) and methanol (E-F) media. The graphs A, C, E represent the light and B, D, F show dark condition.



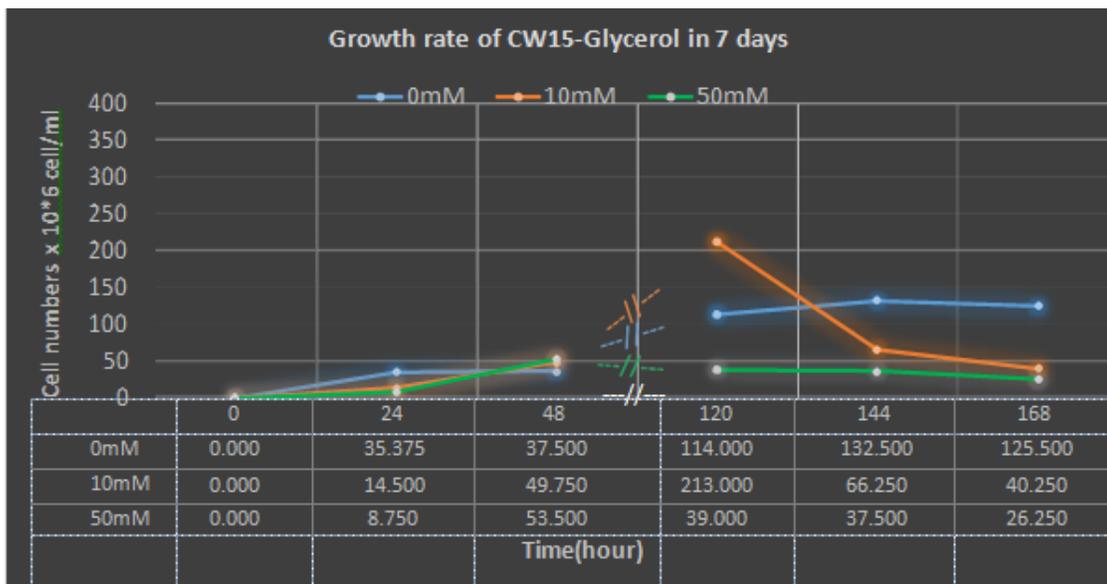
Concentration	Growth rate cells $\times 10^4$ per hour	Divisions per hour	Generation time
0mM	6.5	0.094	10.63
10mM	5.3	0.076	13.15
50mM	8.8	0.127	7.87



Concentration	Growth rate cells $\times 10^4$ per hour	Divisions per hour	Generation time
0mM	4.9	0.071	14.08
10mM	2.7	0.039	25.64
35mM	2.5	0.036	27.77

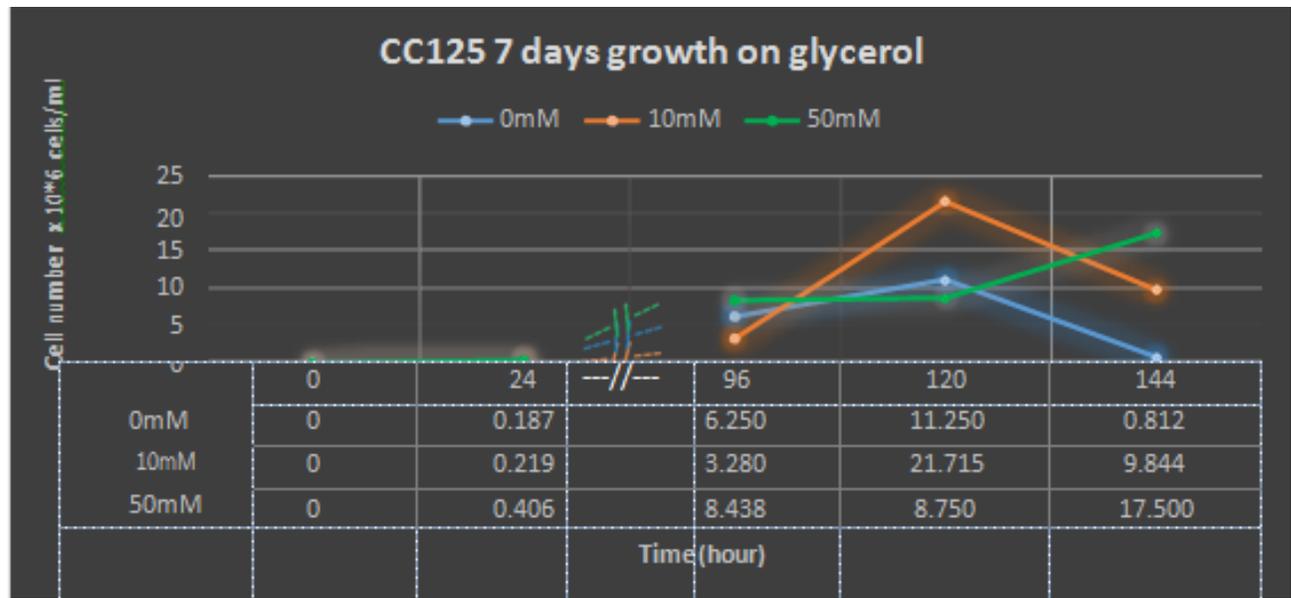


Concentration	Growth rate cells $\times 10^4$ per hour	Divisions per hour	Generation time
0mM	3.4	0.049	20.40
10mM	2.0	0.028	35.71
50mM	1.2	0.017	58.82

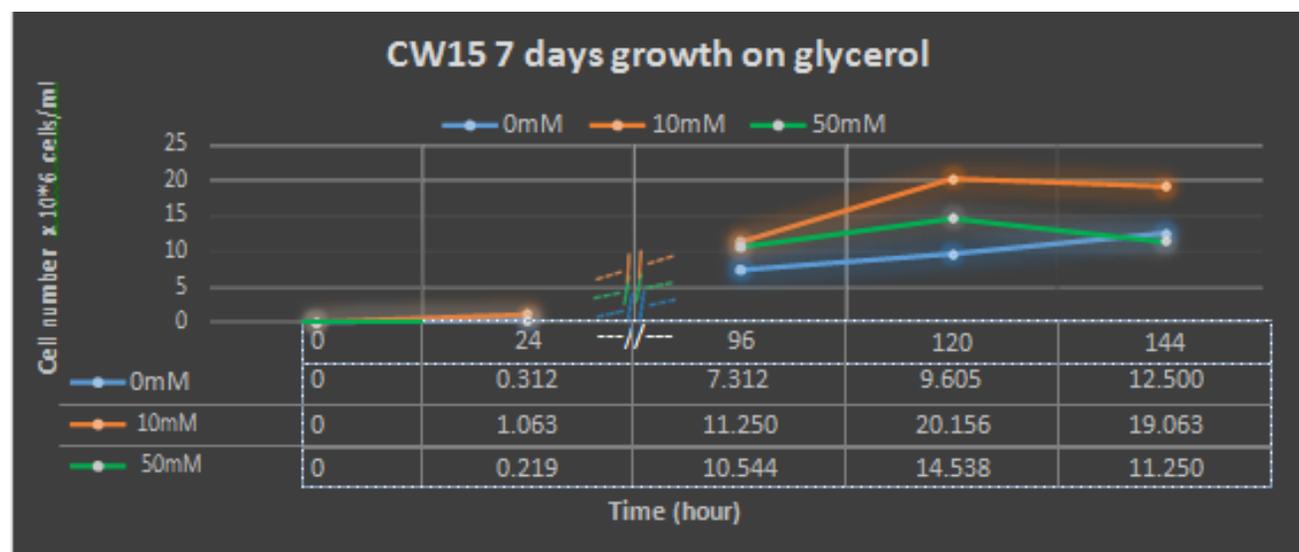


Concentration	Growth rate cells $\times 10^4$ per hour	Divisions per hour	Generation time
0mM	0.5	0.007	143
10mM	5.2	0.075	13.3
50mM	7.5	0.108	9.2

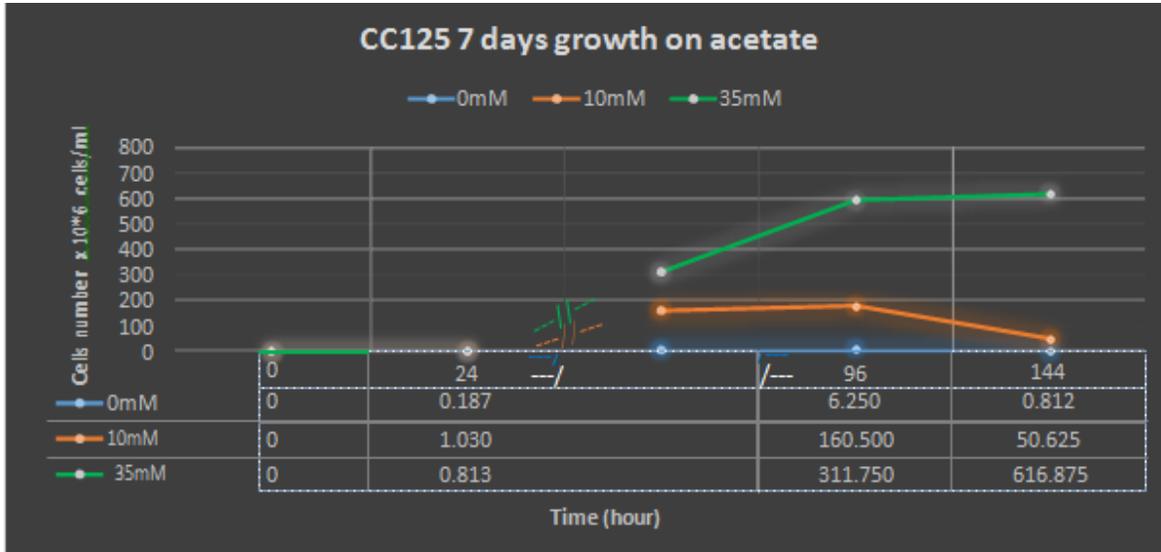
Figure 3. Comparison of 7 days CC125 and CW15 growth on 24 wells micro plate in light condition ($38 \mu\text{E m}^{-2}\text{s}^{-1}$) on three Acetate and Glycerol concentration (0, 10, 35 mM) and (0, 10, 50 mM) respectively. The sign (-/-/-) indicates a data gap between day 2 (48 h) and day 5 (120 h). Blue liner graph indicates phototrophic growth. The orange and green liner graph respectively shows use of 10 and 50 mM glycerol in Figure 3D).



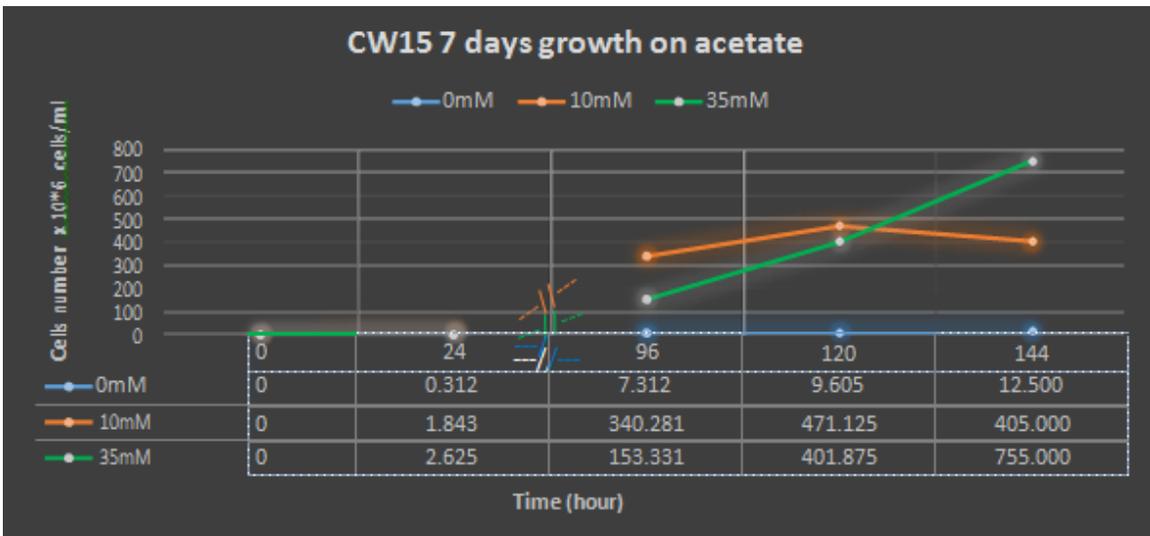
Concentration	Growth rate cells $\times 10^4$ per hour	Divisions per hour	Generation time
0mM	2.3	0.033	30
10mM	7.8	0.113	8.85
50mM	2.8	0.040	25



Concentration	Growth rate cells $\times 10^4$ per hour	Divisions per hour	Generation time
0mM	1.1	0.017	58.823
10mM	2.4	0.034	29.411
50mM	1.3	0.018	55.555



Concentration	Growth rate cells x10 ⁴ per hour	Divisions per hour	Generation time
0mM	2.3	0.033	30
10mM	0.4	0.005	200
35mM	2.6	0.037	27



Concentration	Growth rate cells x10 ⁴ per hour	Divisions per hour	Generation time
0mM	1.1	0.017	59
10mM	1.3	0.018	55
35mM	4.0	0.057	17.5

Figure 4. Comparison of 7 days CC125 and CW15 growth in tissue culture flasks in 38 $\mu\text{E m}^{-2}\text{s}^{-1}$ light condition on three Acetate and Glycerol concentration (0, 10, 35 mM) and (0, 10, 50 mM) respectively. The sign (- - -/-) indicates a data gap between day 1 (24 h) and day 4 (96 h). The low growth rate of CC125 on 10mM acetate (0.004) is probably because the lack of data in exponential phase (between day 1 and 4) and the 0.004cells/hour possibly is a growth rate when cells are entering to the beginning of the stationary phase. Blue liner graph indicates phototrophic growth (The orange and green liner graph respectively show use of 10 and 50 mM glycerol in Figure 4B).

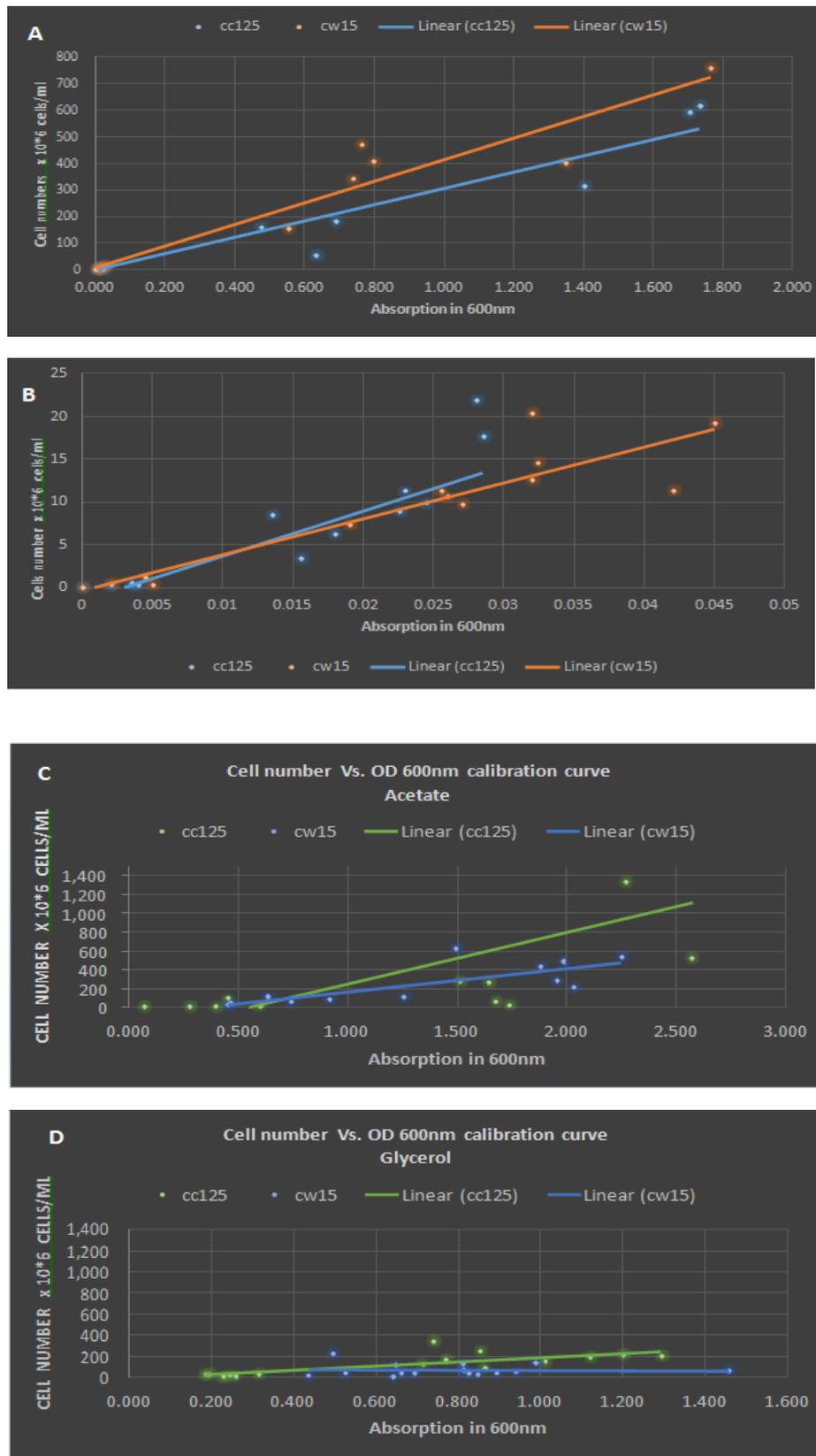
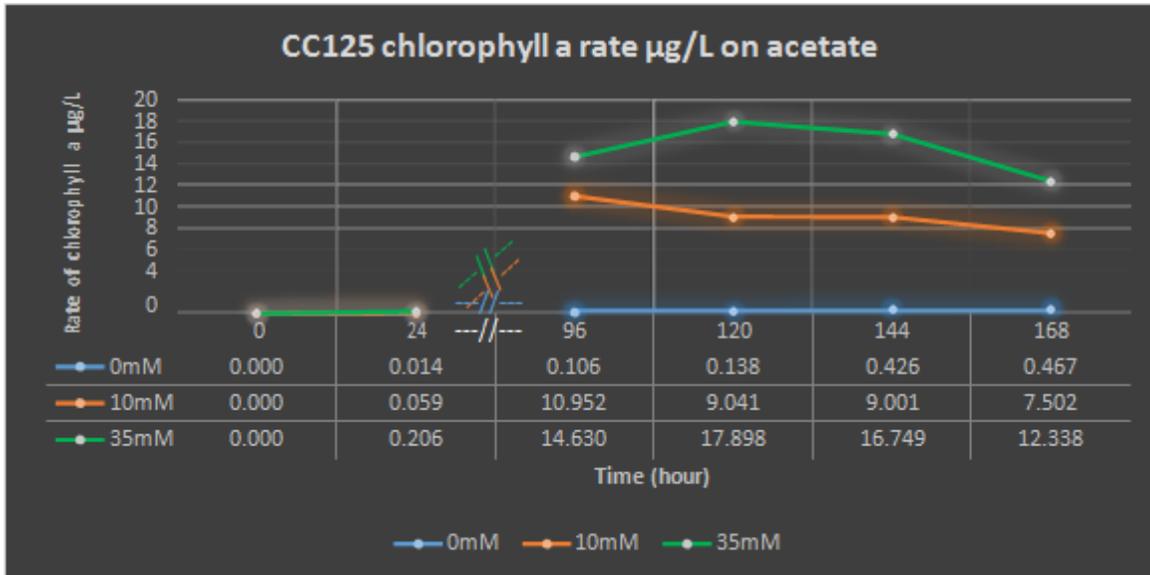
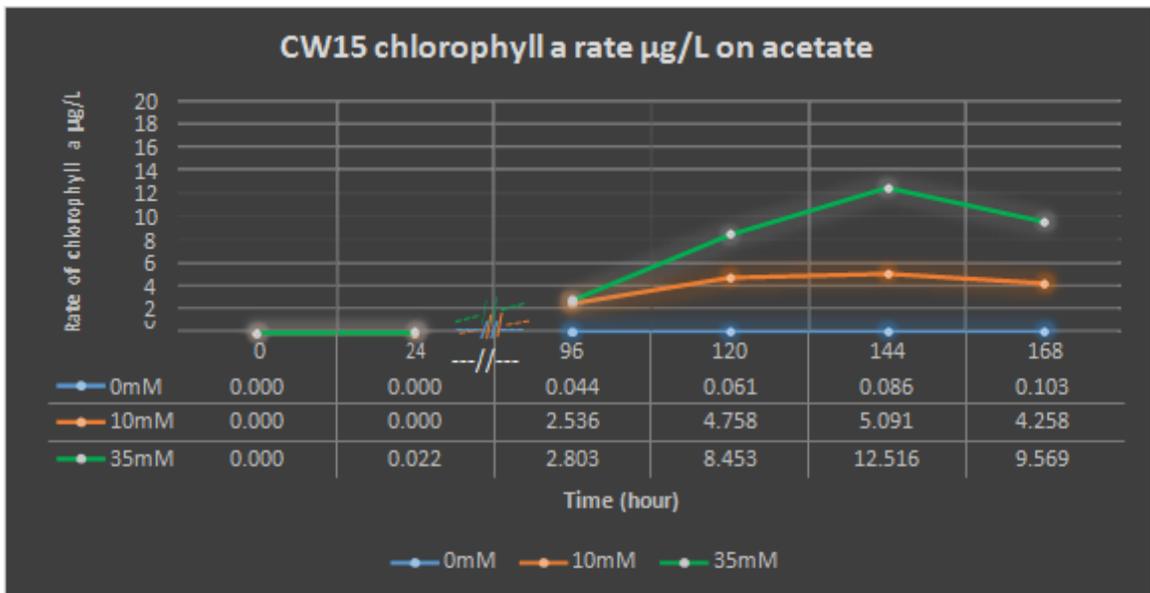


Figure 5. Calibration curve between Absorption n 600 nm and cells number. A –B represent the data for tissue culture flasks, C-D represent the data for micro plate culture. A and C are calibration in acetate. B and D are in glycerol.



Concentration	Growth rate per hour $\mu\text{g.L}^{-1}.\text{h}^{-1}$
0mM	0.046
10mM *	0.151*
35mM	0.008

CW15 chlorophyll a rate $\mu\text{g/L}$ on acetate



Concentration	Growth rate per hour $\mu\text{g.L}^{-1}.\text{h}^{-1}$
0mM	0.014
10mM	0.026
35mM	0.046

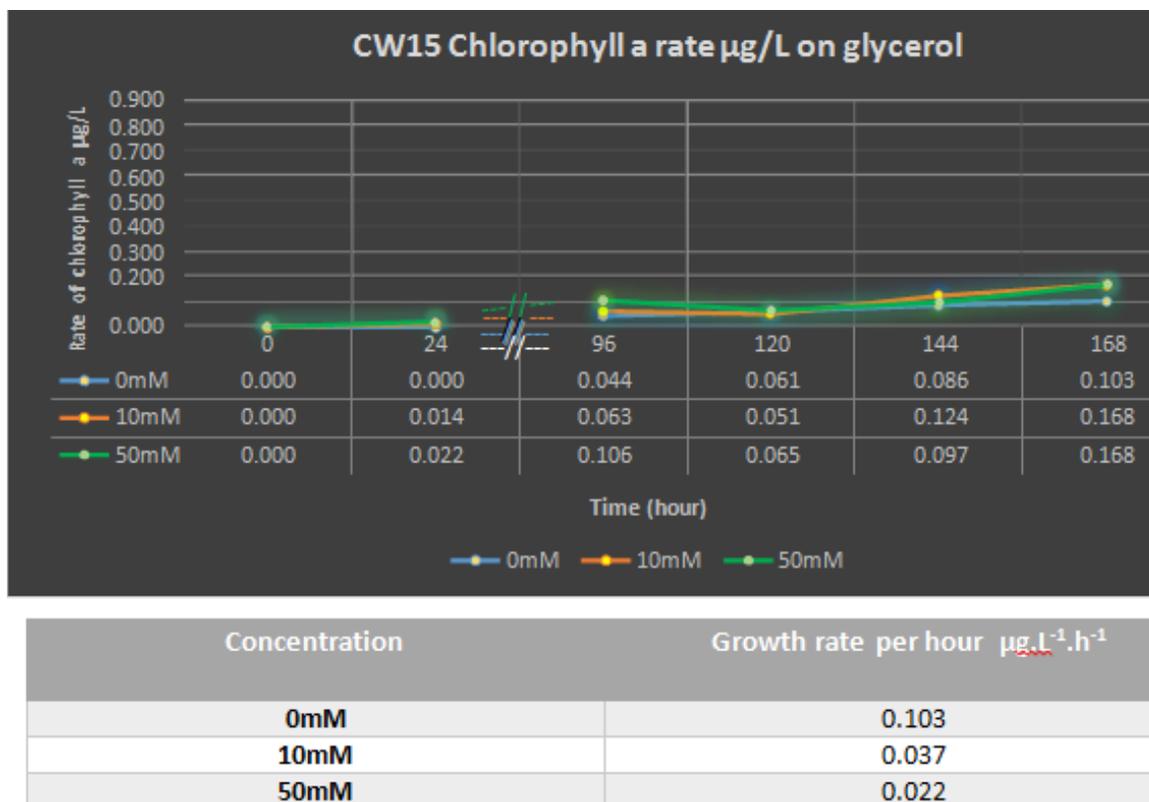
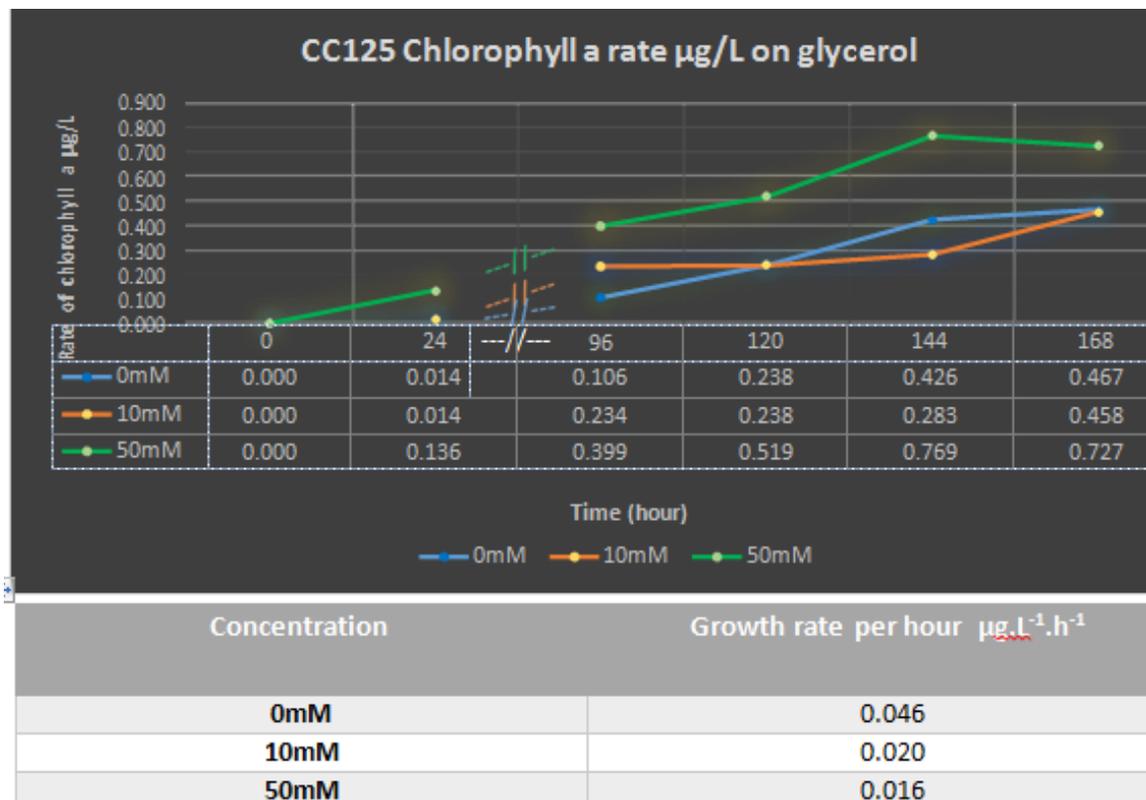


Figure 6. Chlorophyll a production rate in CC125 and CW15 on various concentrations of acetate and glycerol feed in tissue culture flasks. The sign (- - -/- - -) indicates a data gap between day 1 (24 h) and day 4 (96 h). Liner green graph in part A and B show the rate chlorophyll production in use of 35 mM.

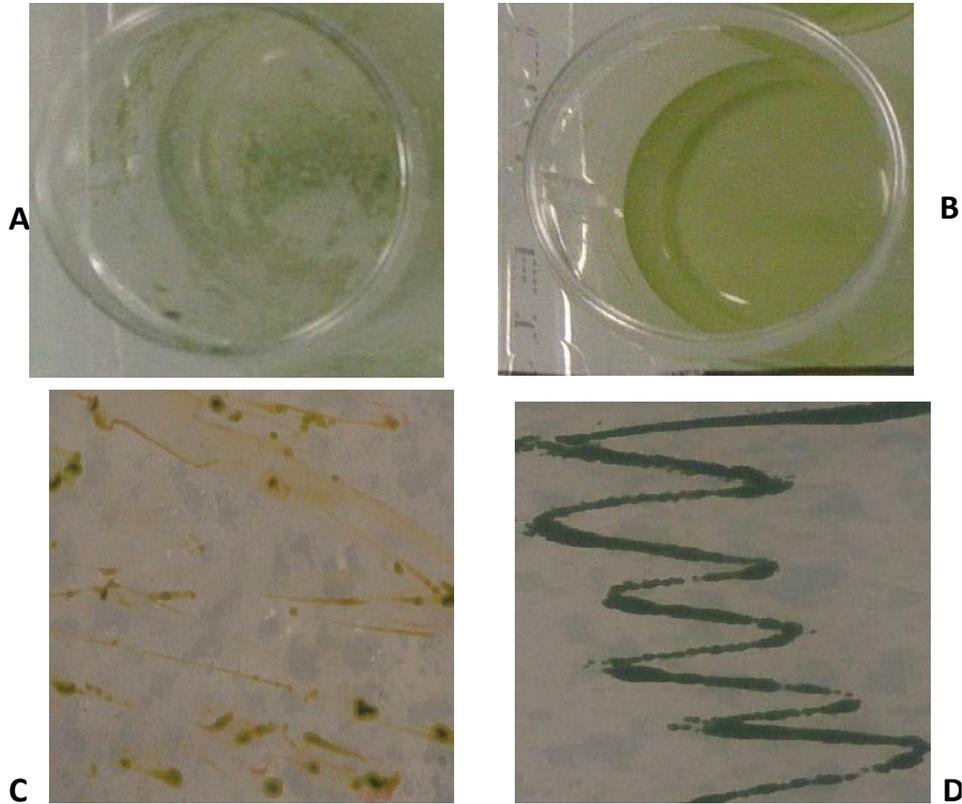


Figure 7. Chlorophyll deficient and cell aggregation in CC125. Picture A and B respectively indicate cells degradation in 100 mM acetate liquid culture under light illumination of $38 \mu\text{E m}^{-2}\text{s}^{-1}$ and normal cells in phototrophic growth condition. Pictures C illustrates a possible deficient in Chloroplast system in utilizing of 100 mM acetate in solid culture under dark condition compare to the phototrophic growth (picture D).

Table 1. Comparison the growth rate of both strains (CC125 and CW15) on acetate and glycerol in TP solid culture under various concentrations and light conditions.

	Acetate	10 mM	20 mM	35 mM	50 mM	100 mM
CC125	Light	++	++	++	++	++
	Dark	-	-	-	+-	+-
CW15	Light	+	+	+	+	+
	Dark	+	+	+	+	+
	Glycerol	10 mM	20 mM	35mM	50mM	100mM
CC125	Light	++	++	++	++	++
	Dark	-	-	-	-	-
CW15	Light	+	+	+-	+-	+-
	Dark	-	-	-	-	-

The ++ is consider as highest growth;- is the lowest rate.

experiment as much as possible and introduce the most optimum industrialized system. Although CW15 had a higher growth rate in micro plate on feed of 50 mM glycerol, as commercial fermenters are much larger than micro plate

and as we got a higher growth rate in use of 10 mM glycerol in tissue culture flasks as an example of bigger culture, so utilizing the 10 mM glycerol commercially and economically is more beneficial as its cost is 1/5 of 50

mM glycerol. The cost of at least minimum volume of 1000 L 10 and 50 mM glycerol solution will be, \$360 and \$1802, respectively.

Susceptibility to bacterial contamination

The rates of algal mixotrophical growth on solid (TPA) and liquid TP cultures were different. On solid TP plates, the growth ratio of CC125 was higher compared to CW15 in both acetate and glycerol (Table 1). In the susceptibility to bacterial contamination experiment that was done on both strains, we found that on TP media CW15 had 28 out of 300 bacterial colonies (10 %) and CC125 had 20 out of 300 colonies (6.6%) whereas on TAP media CW15 and CC125 got 23 out of 300 (7.6%) and 9 out of 300 (3%) bacteria colonies, respectively. On TSB plates, however, solely 2 out of 300 (0.6%) and 1 out of 300 (0.3%) of bacteria colonies were seen on CW15 and CC125 in orderly fashion. This can suggest that the lack of cell wall would increase the chance of bacterial contamination almost 2 times more than the wild type with its cell wall as a barrier.

Moreover, the rate of fungal contamination of CW15 on TSB plates was much higher than CC125 compared to other TP and TAP media cultures.

Thus, one possibility could be that it was as a result of the negative effect of bacteria on growth of CW15 on solid culture. Hence, when cells are introduced in a soluble medium, researchers should not always expect to see the same behavior as to when such cells were in solid media. In shaking liquid culture, there is a possibility that micro gradients are introduced in a watery atmosphere and therefore a lower potential for neighbor effects and bacteria interactions will occur compared to solid media.

Further discussion and future applications

The potential of micro algae as laboratory tool in cosmetic industries (Potvin and Zhang, 2010), drug designing (Bumbak et al., 2011; Milledge, 2010) and recombinant proteins or as a renewable energy source specifically in biofuel production make it an interesting microorganism to invest more attention and effort on its growth and yield (Chisti, 2007; Stephens et al., 2010; Wijffels and Barbosa, 2010; Hempel et al., 2010). So far we showed that mixotrophically growth of CC125 and CW15 on 35mM acetate was the optimum way for faster growth and biomass rate. By knowing that each algal strain behave differently in different media and conditions and there was not any fixed pathway so far therefore a need for further researches and experiments is vital to confidently propose a system with highest and fastest biomass production.

In theme of biofuel one possible application for future purposes can be use of organic (acetate) and inorganic carbon (bicarbonate) in TP media to see its effect on both

growth and lipid production.

As it is in general acceptance that for commercial purposes inorganic carbons are needed and as (Gardner et al., 2012; Gardner et al., 2013) have shown that bicarbonate as a lipid trigger can increase the yield of lipid production such as triacylglycerol (TAG) in some microalgae species (Gardner et al., 2012; White et al., 2012) thus it can worth to determine the outcomes of our experiment (use of 35 mM acetate for faster growth) to other literatures results as a new application to find out the possible rate of lipid production specifically in CW15 that lacks cell wall. The absent of cell wall in CW15 not only has shown a faster growth but also can speed up the cultivation process. What also we really interested in to examine in our future experiment is correlating the effect of various light intensity and temperature (Ras et al., 2013; Spreitzer et al., 1998; Falk et al., 2006) on growth and lipid (TAG) production of our used algal cells (CW15 and CC125).

Moreover, as the rate of bacterial contamination was higher in CW15 than CC125 due to the absence of its cell wall, one interesting application could be the use of bacterial 16srRNA probes to investigate a possible interaction between CW15 and bacteria and the role of bacteria in rate of biomass. The use of this small ribosomal ribonucleic acids subunit (16 srRNA) still is one of the precise method for identification of bacteria as they are conserve molecules and present in all bacteria thus for identification of different bacteria still the recognition system will remain fixed (Ludwig et al., 1998).

Conclusion

The potentiality of microalgae to substitute as renewable energy source to some conventional ways made it interesting. So far micro algae has been cultivated in various conditions (phototrophic, mixotrophic and heterotrophic), and nutritious feed, but each has some merit and demerit points. Introducing algal cells in phototrophic condition minimizes the growth rate, but its advantage is to also decrease the possibility of bacterial contamination. Mixotrophic condition can boost the algal growth and yield due to use of addition of external carbon source and light simultaneously, but also can encourage bacteria into the system due to the existed carbon source. On the other hand, in heterotrophic conditions although the cost of experiment clearly is less than both mixotrophic and phototrophic conditions, because the created dark atmosphere in addition of external carbon is strongly a suitable condition for bacterial and fungus growth the chance of algal yield and growth also will decrease. Moreover, solely some species of algae have capability to grow on dark condition. Despite the advantage and disadvantage of each growth condition our results suggested that acetate was the most effective organic carbon source among here tested carbon sources and the optimal concentration

for *C. reinhardtii* CC125 (wild type) and CW15 (cell wall mutants) to grow quicker was use of 35 mM acetate mixotrophically.

Also, another recent study done by Moon et al. in 2013 showed that among acetate, glucose, glycerol or sucrose, mixotrophically growth of *C. reinhardtii* on acetate was the optimum way in terms of growth and lipid yield. Also, use of 35 mM acetate in 1000 L fermenter is economically even cheaper than use of the alternative carbon source (10 mM glycerol), being \$327 and 363, respectively.

Even though in our experiment a minimum growth rate was seen in feed of both *C. reinhardtii* strains (CC125 and CW15) on glycerol culture (Cerón García et al., 2000) has shown that the use of 0.1M glycerol with light intensity of $165 \mu\text{E m}^{-2}\text{s}^{-1}$ let *Phaeodactylum tricornutum* grow 74% faster than phototrophic condition.

Comparatively to the wild type (CC125), our experiment suggests that the lack of cell wall in CW15 possibly decrease the digesting process time and this can be the reason for a faster growth rate (cells or chlorophyll a) per hour in liquid culture. However, in absence of antibiotics in media the chance of bacterial contamination is approximately 2 times higher in CW15 compare to CC125 that has cell wall as a barrier.

Though the cells morphology data shows that a single algae cell in both CC125 and CW15 has a ring like shape with an approximate same size of 50 μ , biotechnologically growing and cultivating CW15 can be preferred since cells in CW15 cells remain separately from each other while in CC125 mostly join together as a colony in a capsulated environment by their cell wall and this means more small sized cells are better and offer a possible higher cultivation product than a massive cell in less numbers.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations

TAP, Tris-acetate phosphate; **TP**, Tris-phosphate; **TPA**, TP+2% agar; **DMR**, digital module R; **TGA**, triglyceride.

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