A nutrient medium for development of cell dense inoculum in mixotrophic mode to seed mass culture units of *Dunaliella salina*

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With 6 figures and 5 tables

**Abstract:** *Dunaliella salina* is cultivated extensively in natural ponds or intensively in race way ponds for β-carotene production. The ponds are seeded with culture inoculum developed in laboratory conditions or in pilot plant. Because of its high light requirement for growth, *D. salina* cultures are very cell dilute. Therefore, a large volume of culture inoculum is required to seed mass culture units. The feasibility of culture of *D. salina* in mixotrophic mode to obtain cell dense inoculum was investigated with an Indian isolate – I3. The constituents of the mineral medium and their concentration were first standardized in growth assays. The optimized mineral medium was supplemented with organic carbon sources – glycerol, sodium acetate and malt extract and organic nitrogen sources – yeast extract and peptone to test for the best results of mixotrophic culture. A mineral medium with 100 mg L⁻¹ potassium nitrate or urea, 0.35 mg L⁻¹ potassium phosphate, 1 ml L⁻¹ trace elements mix (Walne’s medium) without borate and 12.5 % NaCl in sea water was found optimal. Malt and yeast extracts in the proportion of 1:3 g L⁻¹ in optimized mineral medium was found to result in cell dense cultures. Mixotrophically cultured inoculum grown photoautotrophically in optimized mineral medium resulted in increased biomass production with higher carotene content than when photoautotrophically cultured. The production cycle decreased by 11 days compared to autotrophic cultures

**Keywords:** β-carotene, cell dense cultures, *Dunaliella salina*, malt extract, mixotrophic culture, seed culture, yeast extract

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Introduction

The commercial culture of *Dunaliella salina* for production of β-carotene throughout the world is now one of the success stories of applied phycology (Ben-Amotz & Avron 1980, Borowitzka et al. 1984, Tafreshi & Shariati 2009). *D. salina* is one of the three micro algal species which is successfully cultivated in open ponds (Brennan & Owende 2010, Harun et al. 2010) because of its requirement of high salinity for growth – the extreme condition being unfavourable to predators. β-carotene from *Dunaliella* is now being produced on a commercial scale in Australia, USA and Israel, and pilot-scale projects are under way in China, Chile, Australia, USA, Spain, Kuwait and Cuba (Suárez et al. 1999, García-González et al. 2003, Tafreshi & Shariati 2006). Rengunathan and Kitto (2009) observed that “*Dunaliella* can engineer economic hope for a better life for billions of coastal people who live within 1000 m from the coastline” of India – a subcontinent with a very long coastline in the tropical belt with ample sunlight for most part of the year that suits algal cultivation.

*Dunaliella salina* is cultured intensively in race way ponds (Borowitzka & Borowitzka 1988a, Ben-Amotz 2004, Del Campo et al. 2007). Seed culture for these ponds is developed indoors in the laboratory. The culture is scaled from a small to high volume through serial transfer. This process requires 30 to 60 days depending on the size of the race way ponds to be seeded and the volume of the seed. In the laboratory, temperature and light are conditioned to favor growth. Thus, laboratory culture is both time consuming and energy intensive. Due to requirement of high light intensity, *D. salina* cultures are very cell dilute (Ben-Amotz 1984, Ben-Amotz & Avron 1990, García-González et al. 2005). To develop microalgal seed culture with high cell density “sequential heterotrophy–dilution–photoinduction” method has been suggested (Fan et al. 2012). Heterotrophy in *D. salina* was reported to be possible but not plausible for mass culturing because of the very slow growth (Gladue & Maxey 1994) and the opinion prevails that *D. salina* is an obligate autotroph (Subba Rao 2009). Recently, mixotrophy – culture in light with an organic carbon source in the medium was tested in *D. salina* (Wan et al. 2011, Kim et al. 2012).

Several studies have been made on the culture medium components and their optimal concentration in *D. salina* (Subba Rao 2009). Taking cues from published literature, we developed an optimal culture medium for *D. salina* for quick development of seed culture through mixotrophy and subsequent increased biomass production in outdoor culture. It could be dubbed a “sequential mixotrophy–dilution–phototrophy culture” method.

Materials and methods

A *Dunaliella salina* strain – I3 (identified through rDNA fingerprint, Suman 2012) isolated from evaporation salt pans was selected for optimization of culture medium. The carotene content per unit culture volume and per cell in I3 strain is higher than
the other strains with smaller cell size in our laboratory repository. First the inorganic mineral medium required for optimal culture under photoautotrophic conditions was developed. The thus optimized medium was fortified with organic carbon and nitrogen source to serve as a medium for mixotrophic culture.

Walne’s medium with 12.5 % NaCl was taken as the base medium for improvisation. Walne’s medium is a semi-defined medium made from sea water (Walne 1970). The main components of the medium are nitrate and phosphate. The concentration range of phosphate used in different media varies only in the range of 0.005 g L$^{-1}$ (Subba Rao 2009). Therefore the concentration of phosphate was not optimized and a concentration of 0.035 g L$^{-1}$ (used in modified Johnson’s medium, Borowitzka & Borowitzka 1988a) was used in the medium. Vitamin mix of thiamine hydrochloride (B 1), biotin (H) and cyanocobalamine (B$_{12}$) is used in Walne’s medium. Borate is used in much higher concentration in Walne’s medium than other media used for culture of Dunaliella. Therefore the following components of the medium were optimized.

A. Best nitrogen source and its optimal concentration among sodium nitrate, potassium nitrate, urea and ammonium chloride;

B. Requirement of micronutrient mix, borate and vitamin mix and

C. Optimal sodium chloride concentration.

In thus optimized mineral medium, different complex nutrients with a major component as carbon or nitrogen: organic carbon sources – sodium acetate, glycerol and malt extract and organic nitrogen sources – yeast extract and peptone at different concentrations were tested.

**Mineral medium**

The inorganic nitrogen sources – sodium nitrate, potassium nitrate and ammonium chloride and organic nitrogen source – urea were tested at four concentrations – 50, 100, 150, 200 mg L$^{-1}$. In the medium with an optimal concentration of the best nitrate source, growth was monitored with and without vitamin mix, trace elements and the major (used in high concentration) trace element borate. Salt concentrations ranging from 10 to 30 % (molarity values of ~2.2, 2.6, 3.0, 3.9, 4.8 and 5.6 M) were tested. A medium with components and their concentration found optimal in these experiments was thus devised and growth of $D. salina$ in this medium was compared to the control (Walne’s with 12.5 % NaCl) was compared.

**Medium with complex nutrients to serve as organic carbon and nitrogen source for promoting growth in mixotrophic mode**

A concentration range between 2–10 g L$^{-1}$ for sodium acetate, 1–10 g L$^{-1}$ for malt extract, 1–5 % for glycerol and 1 to 4 g L$^{-1}$ of yeast extract and peptone were tested. The yeast and peptone combinations were tested with the complex nutrient which was found as the best organic carbon source for growth. The utilization of malt extract in the nutrient medium was monitored through anthrone test (Hansen & Moller 1975).
An aliquot (2 ml) of culture was drawn on alternate days after inoculation with alga until the end of the experiment. The algal cells were pelleted down and the supernatant culture medium was used for estimation of sugar through anthrone test (Hansen & Moller 1975).

**Experimental setup**

The algal cultures were established in 250 ml conical flasks with 150 ml of culture medium. The inoculum (20 % of the volume of the culture medium) for the experiments was drawn from cultures in Walne’s medium at the mid exponential stage with a density of 2 x 10^5 cells ml^-1. In experiments for testing the optimal sodium chloride concentration, the cultures to serve as inoculum for the test NaCl concentration were first set up in the test concentration and the habituated culture was used for experiments. The mouth of the culture flasks was covered with non absorbent cotton plugs which allow gaseous exchange but prevent microbial contamination. The cultures were maintained at 25°C ± 2°C, 150 μmol photons m^-2 s^-1 (8.1 Klux) with a 12/12h light/dark cycle. The culture flasks were manually agitated twice a day. The pH of the culture was monitored throughout the experiments. It ranged between 7.9 to 8.2.

**Assessment of growth**

Growth was assessed from cell counts made on consecutive days after set up of the experiment. The culture drawn was preserved in 20 μl of FAA (1:1 formalin: glacial acetic acid) until counts were made. The preserved aliquots were used to enumerate cell numbers with a Neubauer haemocytometer. For each sample, counts were made twice and the mean cell number was calculated. From these mean cell numbers on different days after inoculation, rate of growth (k) was computed from which division rate (μ), and generation time (G) were derived (Furnas 2002). The chlorophyll and carotenoid content was estimated spectrophotometrically (Jeffrey & Humphrey 1975, Boro-witzka & Siva 2007).The experiments were set up in duplicate and repeated thrice. The mean and standard error of the replicates was calculated. Tukey HSD test was done to compare the results of different treatments. Tukey HSD statistical test is a single-step multiple comparison procedure. It is used in conjunction with ANOVA to find means that are significantly different from each other. Tukey’s test compares the means of every treatment to the means of every other treatment; that is, it applies simultaneously to the set of all pairwise comparisons and identifies any difference between two means that is greater than the expected standard error. The ANOVA and HSD [0.05] – the absolute (unsigned) difference between any two sample means required for significance at 0.05 probability in each of the computations are given. The assistat statistics assistance software (Silva & Azevedo 2009) was used for statistical analysis.
Mass culture

The seed culture for mass culture was developed both in the optimized mineral medium and mixotrophic medium through gradual upscaling from 10 ml to 250 ml to 2 L in glass flasks followed by 3 L and 5 L pet jars in the laboratory. They were further up scaled to 20 L pet jar to 80 L, 50 L and 1000 L FRP (fibre reinforced plastic) tanks in a glass house. At all steps of upscaling, algal culture in a volume constituting 20% of the total culture volume was inoculated. The mouth of the pet jars was closed with loosely turned in screw caps. The screw caps were perforated (with a hot iron needle) to facilitate aeration. The FRP tanks were left open. The containers were filled to 70% of their volume with medium (Lee & Tamaru 1993, Lavens & Sorgeloos 1996). No autoclaved water was used in medium preparation from 1 L volume. The cultures in volumes above 20 L were continuously aerated with a spurger – an air hose pipe with air stone. Further, cultures in the 1 ton tank were manually mixed by rotating the culture medium with a 2”diameter meter length PVC (polyvinyl chloride) pipe for five min thrice every day. The seed culture developed in mixotrophic mode (up to 150 L) was transferred to: a. mixotrophic medium, b. optimized mineral medium. As a control, seed culture developed in autotrophic mode was transferred to optimized mineral medium. In the one ton FRP tanks, pH and salinity of the cultures were monitored every day. Salinity was measured with a salinity refractometer (MASTER-S/MillM, ATAGO JAPAN). Sea water was added to the culture medium to compensate for the evaporated water and restore salinity to 12.5%. The growth in the cultures was followed by taking cell counts in an aliquot of the culture collected after manual agitation of the culture. The cultures reached the end of exponential phase by 26th day. At this stage, the salinity of the medium was increased to ~30% (300 ppt) to stress the cells and induce accumulation of β-carotene. Crude crystal salt (from evaporation salt pans) was used and the tanks were placed under direct sunlight (76–82 Klux, ~10 hours light duration) for three days by which time the cultures turned orange to brown. The mass culture schedule lasted for ~60 days during which time the physical conditions in the glass house were as follows: temperature: 26–32°C day /16–22°C night, light: 33–58 Klux and duration: ~10 h.

Harvesting

Ferric chloride (1 g L⁻¹) solution of the volume approximated from flocculation in small culture (equivalent to 0.8 to 1 g L⁻¹ of the algal culture depending on its density) was dispensed into the culture in 1 ton tank and shaken thoroughly with 2”diameter and meter length PVC (polyvinyl chloride) pipe. The flocculated culture was left overnight for settling of the biomass. The clear supernatant in the culture tank was decanted off by pumping out with rubber hose pipe fitted to a 0.5 hp motor. The remaining culture was filtered through a 100 micron pore size filter nytex cloth (PLANKTON NET FABRIC, Nytex netting). The resultant biomass slurry was washed once with 10 L of isotonic ammonium formate (0.5 M) to remove salts with-
out causing the cells to burst (Lavens & Sorgeloos 1996) and the wet biomass was dried in a hot air oven for 12 h at 50°C.

**Qualitative and quantitative estimation of pigments**

Qualitative and quantitative analysis of pigments in the harvested biomass both in wet and dry condition was done through HPLC with Agilent 1200 HPLC system equipped with quaternary pump, auto injector, Peltier column thermostat, temperature controlled auto sampler and Chemstation software. Pigments were detected with a diode array detector at 450 and 665 nm wavelength (20 nm bandwidth was used in both cases). Carotenoids and xanthophylls were detected and quantified at 445 nm (Van Heukelem & Thomas 2001) and chlorophyll \( \alpha \) was quantified at 665 nm. An injector program was optimized to deliver sample extract and buffer composed of 28 mM aqueous tetrabutyl ammonium acetate (TBAA) (AR Grade, Fluka) at pH 6.5 and methanol (GC assay 99.7 % pure, Merck) in a 90:10 ratio. The sample extract and buffer was mixed automatically within the sample loop, which enabled effective retention of early eluting chlorophylls and lessened peak distortion.

**Results**

Maximum growth was observed in cultures at a concentration of 100 mg L\(^{-1}\) of nitrate from all nitrogen sources except ammonium chloride in which 50 mg L\(^{-1}\) was found optimal (Fig. 1). The growth in medium with 100 mg L\(^{-1}\) of both potassium nitrate and urea were found similar and better than in sodium nitrate and ammonium chloride. At this concentration of nitrogen source, the ANOVA and HSD values for the different growth parameters were: G (Generation time) \( \text{df} = 3, F = 99.12, P<0.001 \), HSD\([.05]\) = 1.48; Max cell no (x 10\(^6\) ml\(^{-1}\)) df = 3, \( F = 51.99, P<0.001 \), HSD\([.05]\) = 8.06 and Division rate (μ).

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**Fig. 1.** Growth parameters of I3 strain of *Dunaliella salina* in Walne’s medium substituted with different nitrogen sources at different concentrations. Numbers on top of bars represent growth rate (μ) – the standard error values ranged between 0.00–0.01. Bars and numbers with the same lower case alphabets in different treatments are similar as deduced from Tukey HSD test. The ANOVA and HSD values for: **Na NO\(_3\)** – G (Generation time): \( \text{df} = 3, F = 641.83, p<0.001 \), Tukey HSD\([.05]\) = 1.41; Max cell no (x 10\(^6\) ml\(^{-1}\)) df = 3, \( F = 107.11, p<0.001 \); Tukey HSD\([.05]\) = 5.19; Division rate (μ): df = 3, \( F = 163.77, p<0.001 \); Tukey HSD\([.05]\) = 0.02; **KNO\(_3\)** – G (Generation time): \( \text{df} = 3, F = 83.73, p<0.001 \); Tukey HSD\([.05]\) = 2.17; Max cell no (x 10\(^6\) ml\(^{-1}\)): \( \text{df} = 3, F = 18.29, p<0.001 \); Tukey HSD\([.05]\) = 5.55; Division rate (μ): df = 3, \( F = 72.25, p<0.001 \); Tukey HSD\([.05]\) = 0.02; **Urea** – G (Generation time): \( \text{df} = 3, F = 1283.62, p<0.001 \); Tukey HSD\([.05]\) = 0.95; Max cell no (x 10\(^6\) ml\(^{-1}\)): \( \text{df} = 3, F = 2.86, p<0.063 \); Division rate (μ): df = 3, \( F = 527.13, p<0.001 \); **NH\(_4\)Cl** – G (Generation time): \( \text{df} = 3, F = 1021.4, p<0.001 \); Tukey HSD\([.05]\) = 0.95; Max cell no (x 10\(^6\) ml\(^{-1}\)): \( \text{df} = 3, F = 1.81, p<0.177 \); Division rate (μ): df = 3, \( F = 202.86, p<0.001 \); Tukey HSD\([.05]\) = 0.02. HSD \([0.05]\) values are not available when K>2 and \( F \) has a significant ratio.
rate (μ): df = 3, F = 107.14, P<0.001. HSD [0.05] value not available because K>2 and F has a significant ratio. For an equal weight of the compound, the amount of nitrogen available from urea is more than that from potassium nitrate because each molecule of urea has two nitrogen atoms while potassium nitrate has one nitrogen.

In medium without trace elements, maximum cell number was reduced to ~3.5 of the control, the generation time was increased by ~20 h and division rate significantly reduced compared to the control (Fig. 2). In the medium without trace elements, the chlorophyll content per cell increased ~11 times and the carotenoid content was also significantly higher than control (Fig. 2). In comparison to the control, in the medium without borate, maximum cell number was significantly higher, the generation time was ~11 h less, the chlorophyll content per cell was ~ double while the carotenoid content per cell was reduced to 1/5th the control (Fig. 2). In medium without vitamin mix, a higher growth rate and ~10 h lesser generation time was observed compared to the control though there was no significant difference in the maximum cell density (Fig. 2). In the medium without vitamin mix chlorophyll a content was similar to control but the carotenoid content was significantly lesser than the control (Fig. 2).
Among the different NaCl concentrations tested, 12.5 % (used in Walne’s medium) was found optimal (Table 1). Growth was reduced as the concentration of NaCl increased (Table 1). In a medium with 10 %, NaCl the maximum cell number attained was lower than in a medium with 12.5 % NaCl, though growth rate was similar at both these concentrations of NaCl (Table 1).

From the above results the optimal mineral medium was formulated as: KNO₃–100 mg L⁻¹; KH₂PO₄–0.35 g L⁻¹; trace element mix (Walne’s) without H₃BO₃–1 ml L⁻¹ and 12.5 % NaCl. Growth in optimized mineral medium was significantly higher than in Walne’s (with 12.5 % NaCl) medium (Table 2). The cost of the optimized mineral medium is 4 US$ per 1000 L compared to 12 US $ for Walne’s medium.

No growth was observed in mineral medium supplemented with glycerol. At all sodium acetate and malt extract concentrations tested, growth was better than in optimized mineral medium (Figs. 3, 4). A concentration of 4 g L⁻¹ of sodium acetate and 6 g L⁻¹ of malt extract were found optimal (Figs. 3, 4). Growth at all concentrations of malt extract was much higher than in medium with sodium acetate (Figs. 3, 4). Anthrone test results indicated a gradual decrease in concentration of malt extract in the medium with a steep decrease observed in samples collected on 6th day after algal
**Table 1.** Growth of I3 strain of *Dunaliella salina* in Walne’s medium with different concentrations of salt (NaCl).

<table>
<thead>
<tr>
<th>NaCl conc</th>
<th>Max cell no x 10^6 ml⁻¹</th>
<th>Chl a max (pg cell⁻¹)</th>
<th>Specific growth rate (μ)</th>
<th>G = 24/μ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% (1.71 M)</td>
<td>4.04 ± 1.52b</td>
<td>2.87 ± 0.01b</td>
<td>0.36 ± 0.02a</td>
<td>67.00 ± 2.74c</td>
</tr>
<tr>
<td>15% (2.56 M)</td>
<td>2.78 ± 1.52c</td>
<td>3.85 ± 0.01a</td>
<td>0.31 ± 0.01b</td>
<td>78.14 ± 1.42d</td>
</tr>
<tr>
<td>20% (3.42 M)</td>
<td>1.80 ± 2.19d</td>
<td>3.00 ± 0.01b</td>
<td>0.26 ± 0.01c</td>
<td>92.18 ± 3.47c</td>
</tr>
<tr>
<td>25% (4.27 M)</td>
<td>1.11 ± 0.92e</td>
<td>0.99 ± 0.04c</td>
<td>0.18 ± 0.01d</td>
<td>135.38 ± 4.06b</td>
</tr>
<tr>
<td>30% (5.13 M)</td>
<td>0.88 ± 3.35f</td>
<td>1.07 ± 0.00c</td>
<td>0.17 ± 0.00d</td>
<td>143.59 ± 2.34c</td>
</tr>
<tr>
<td>12.5% (2.13 M)</td>
<td>4.23 ± 4.49a</td>
<td>2.62 ± 0.00b</td>
<td>0.35 ± 0.00a</td>
<td>67.82 ± 0.91e</td>
</tr>
</tbody>
</table>

ANOVA and Tukey HSD

df = 5, df = 5, df = 5, df = 5,
F = 3026.53, F = 62.41,
df<0.001; HSD[.05] = 11.79
HSD[.05] = 0.58
F = 252.27, F = 390.99,
df<0.001; HSD[.05] = 0.02
HSD[.05] = 7.5

HSD[.05] = the absolute (unsigned) difference between any two sample means required for significance at a 0.05 probability

± values represent SE

Means with the same superscript alphabet within a column are similar with the Tukey HSD value being not significant at 0.05 probability

**Table 2.** Growth of I3 strain of *Dunaliella salina* in optimised mineral medium in comparison to Walne’s medium.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Max cell no x (10^6 ml⁻¹)</th>
<th>Specific growth rate (μ)</th>
<th>G=24/μ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walne’s</td>
<td>1.86 ± 0.01b</td>
<td>0.31 ± 0.004b</td>
<td>74.93 ± 0.32a</td>
</tr>
<tr>
<td>Optimized mineral</td>
<td>2.01 ± 0.02e</td>
<td>0.43 ± 0.002a</td>
<td>55.14 ± 0.44b</td>
</tr>
<tr>
<td>ANOVA and Tukey HSD</td>
<td>df = 1, F = 33.03, p&lt;0.001; HSD[.05] = 11.79</td>
<td>df = 1, F = 648, p&lt;0.001; HSD[.05] = 0.58</td>
<td>df = 1, F = 1271.14, p&lt;0.001; HSD[.05] = significant F-ratio.</td>
</tr>
</tbody>
</table>

HSD[.05] = the absolute (unsigned) difference between any two sample means required for significance at 0.05 probability

Means with the same superscript alphabet within a column are similar with the Tukey HSD value being not significant at 0.05 probability

± values represent SE

*Optimized mineral medium: KNO₃–100 mg L⁻¹, KH₂PO₄–0.35 mg L⁻¹, trace elements (stock of Walne’s) without H₃BO₃–1 ml L⁻¹, 12.5 % NaCl
Fig. 4. Growth of 13 strain of *D. salina* in mixotrophy mode in optimized mineral medium supplemented with different concentrations of malt extract as an organic carbon source. Numbers on top of bars represent division rate (μ) – the standard error for all values was 0. Bars and numbers with the same lower case alphabets in different treatments are similar as deduced from Tukey HSD test. The ANOVA and HSD values for G (Generation time): \(df = 6, F = 3999.14, p<0.001; HSD[.05] = 0.58\); Max cell no (x 10⁶ ml⁻¹): \(df = 6, F = 4847.92, p < .001; HSD[.05] = 5.91\); Division rate (μ): \(df = 6, F = 996.61, p<0.001; K>2, a significant F-ratio\); Chlorophyll *a* max (pg cell⁻¹): \(df = 6, F = 258.09, p <0.001; HSD[.05] = 0.06\); Carotene max (pg cell⁻¹): \(df = 6, F = 1077.52, p<0.001; HSD[.05] = 0.06\).
inoculation (results not shown). With a raise in concentration of malt extract, along with *D. salina*, an increased growth of small rod shaped cells was observed. Cultures with these cells had a pink tinge. These cells were identified (from the HPLC pigment profile) as *Halobacterium* spp. – a halophilic archaea (unpublished results). With yeast extract, a concentration of 1 g L\(^{-1}\) was found optimal (Fig. 5). In mineral medium supplemented with peptone at all concentrations tested, bacterial contamination was observed. These bacteria were coiled (unlike the rod shaped *Halobacterium* spp.) and the cultures became turbid.

Malt extract was chosen to test in combination with organic nitrogen sources for formulation of medium for mixotrophy. To obtain archaea and bacterial free cultures, lower than optimal concentrations of malt extract and organic nitrogen were tried in different proportions (Fig. 6). Growth was better than in mineral medium in all media supplemented with malt extract and organic nitrogen source (Fig. 6). Best growth was observed when medium was substituted with malt extract, peptone and yeast extract in the ratio of 1:1:3 g L\(^{-1}\) (Fig. 6). However, presence of peptone promoted copious

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**Fig. 5.** Growth of I3 strain of *D. salina* in mixotrophy mode in optimized mineral medium supplemented with different concentrations of yeast extract, a complex organic nitrogen source. Numbers on top of bars represent division rate (μ) – the standard error values ranged between 0.00–0.01. Bars and numbers with the same lower case alphabets in different treatments are similar as deduced from Tukey HSD test. The ANOVA and HSD values for Max cell no (x 10\(^6\) ml\(^{-1}\)): df = 7, F = 408.73, p<0.001; HSD[.05] = 5.82; G (Generation time): df = 7, F = 286.25, p<0.001; HSD[.05] = 0.4; Division rate (μ): df = 7, F = 114.21, p<0.001; K>2, significant F-ratio.
Fig. 6. Growth of I3 strain of *D. salina* in mixotrophy mode in optimized mineral medium supplemented with malt extract (M) as an organic carbon source and peptone (P) and or yeast extract (Y) as complex organic nitrogen source. MPY 1:1:3* is medium with malt extract, peptone and yeast extract added to sea water with 12.5% NaCl added. Numbers on top of bars in the top figure represent division rate (μ) – the standard error for all values was 0.01. Bars and numbers with the same lower case alphabets in different treatments are similar as deduced from Tukey HSD test. The ANOVA and HSD values for dry biomass (mg−1): df = 7, F = 351.88, p<0.001; HSD[.05] = 0.29; Max cell no (x 10⁶ ml⁻¹): df = 7, F = 3864.52, p<0.001; HSD[.05] = 5.55; Division rate (μ): df = 7, F = 82.93, p<0.001; HSD[.05] = 0.03; Chlorophyll *a* max (pg cell⁻¹): df = 7, F = 84.66, p<0.001; HSD[.05] = 0.1; Carotene max (pg cell⁻¹): df = 7, F = 306.67, p<0.001; HSD[.05] = 0.02.
bacterial (coiled bacteria) growth. A combination of malt and yeast extract in 1:3 g L\(^{-1}\) proportion was found optimal for growth of \(D. \text{salina}\) (Fig. 6). No archaea or bacterial cells were observed in these cultures. The presence of bacteria in the cultures was checked by plating an aliquot of the culture on TYG (tryptophan, yeast, glucose with 1.5 % agar) medium (Ferris & Hirsch 1991) and through observation of cultures under a microscope at a magnification of 1500X. The 1:3 g L\(^{-1}\) proportion of malt and yeast extract in sea water (without any mineral fortification) with 12.5 % NaCl also promoted much better growth than optimized mineral medium (Fig. 6). The growth rate in such cultures was also much higher than the control (Fig. 6). These cultures reached exponential phase of growth three days earlier than the control autotrophic cultures.

In mass culture, growth rate and biomass productivity in both the mixotrophic and mixo-autotrophic cultures was much higher when compared to autotrophic cultures (Table 3). In mixo-autotrophic cultures the generation time was \(\sim 45\ %\) lesser than the photoautotrophic cultures (80 h vs 57 h – Table 3) which means the cultures reached the end of exponential phase of growth \(\sim 6\) days earlier. Cell size was uniform in these cultures and they turned orange two days earlier than control.

Six pigments: chlorophyll \(\text{a}\) and \(\text{b}\), \(\beta\)-carotene, lutein, violaxanthin, zeaxanthin and neoxanthin could be identified in the HPLC profiles of the wet biomass (Online Resource 1, 2). On drying the biomass, chlorophyll \(\text{a}\), violoxanthin and neoxanthin were lost (Online Resource 1, 2, Table 4). Except lutein and neoxanthin the other pigments were quantified because standards were available. Cells cultured in mixotrophic mode contained the highest concentration of all pigments that were quantified (Table 4).

The cost-benefit ratios of the three modes of mass culture tested are computed (Table 5). The cost of mixo-autotrophic cultures was 8.7 % (0.6 US $ per 1000 L cul-

### Table 3. Growth and biomass yield of I3 strain of \(D. \text{salina}\) mass cultured in different nutritional modes.

<table>
<thead>
<tr>
<th>Culture mode</th>
<th>Max cell no x 10(^6) ml(^{-1})</th>
<th>Specific growth rate ((\mu))</th>
<th>G = 24/(\mu) (h)</th>
<th>Dry biomass (g L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixotrophy</td>
<td>2.45 ± 1.97(^a)</td>
<td>0.61 ± 0.01(^a)</td>
<td>39.56 ± 0.35(^c)</td>
<td>2.06 ± 0.01(^a)</td>
</tr>
<tr>
<td>Mixo to auto(^a)</td>
<td>1.97 ± 1.20(^b)</td>
<td>0.42 ± 0.01(^b)</td>
<td>56.05 ± 0.49(^b)</td>
<td>1.77± 0.04(^b)</td>
</tr>
<tr>
<td>Autotrophy</td>
<td>1.68 ± 1.17(^c)</td>
<td>0.31 ± 0.01(^c)</td>
<td>79.43 ± 0.49(^a)</td>
<td>1.25± 0.05(^c)</td>
</tr>
<tr>
<td>ANOVA and Tukey HSD</td>
<td>F = 675.49, p&lt;0.001; F = 503.52, p&lt;0.001;</td>
<td>HSD[.05] = 5.8</td>
<td>HSD[.05] = 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F = 1997.26, p&lt;0.001; F = 94.21, p&lt;0.001;</td>
<td>HSD[.05] = 1.75</td>
<td>HSD[.05] = 0.18</td>
<td></td>
</tr>
</tbody>
</table>

HSD[.05] = the absolute (unsigned) difference between any two sample means required for significance at 0.05 probability

Means with the same superscript alphabet within a column are similar with the Tukey HSD value being not significant at 0.05 probability

\(\pm\) values represent SE

\(^a\)Seed culture in mixotrophy and mass culture in autotrophy
Table 4. Pigments estimated through HPLC in I3 strain of *Dunaliella salina* mass cultured in different trophic modes.

<table>
<thead>
<tr>
<th>Pigments</th>
<th>Mixotrophy&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mixo to Autotrophy&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Autotrophy&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wet biomass (mg L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>Dry biomass (pg cell&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>Wet biomass (mg L&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>1.12</td>
<td>0.74</td>
<td>0.29</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>2.05</td>
<td>1.37</td>
<td>0.35</td>
</tr>
<tr>
<td>chl b</td>
<td>16.92</td>
<td>11.28</td>
<td>3.75</td>
</tr>
<tr>
<td>chl a</td>
<td>29.61</td>
<td>19.74</td>
<td>7.92</td>
</tr>
<tr>
<td>β-carotene</td>
<td>112.01</td>
<td>74.67</td>
<td>56.71</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mixotrophy: culture in medium with organic carbon and nitrogen sources – malt extract : yeast extract – 1:3 g L<sup>-1</sup> in optimized mineral medium

<sup>b</sup>Seed culture in mixotrophic mode and mass culture in optimized mineral medium

<sup>c</sup>Culture in optimized mineral medium

For cultures in mixotrophic and mixo to autotrophic mode, wet biomass was analysed.

Table 5. Cost – benefit estimates (for 1000 L culture) of different mass culture methods of *Dunaliella salina* with the optimized mineral medium (autotrophic) and malt and yeast extract (3:1) in optimized mineral medium (Mixotrophic).

<table>
<thead>
<tr>
<th>Culture mode</th>
<th>Medium cost (US $)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% increase over autotrophic culture</th>
<th>Corrected cost (US $)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Days saved&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixotrophic</td>
<td>26.4</td>
<td>65</td>
<td>313</td>
<td>13.2</td>
</tr>
<tr>
<td>Mixo–autotrophic&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.6</td>
<td>42</td>
<td>108</td>
<td>3.96</td>
</tr>
<tr>
<td>Autotrophic</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Seed culture mixotrophic and mass culture autotrophic (the optimized mineral medium is seeded with 20% its final volume with mixotrophically cultured algal inoculum)

<sup>b</sup>With food grade (Chaitanya Agro biotech Pvt. Ltd. India) malt and yeast extract. Cost computed @ of 1 US $ = 60 Indian rupees.

<sup>c</sup>The cost is corrected for increased (17.5% more than autotrophic culture) biomass and β-carotene productivity (double the content per unit mass compared to autotrophic culture)

<sup>d</sup>For production cycle compared to autotrophic culture

...
Discussion

In this work, potassium nitrate was found to be better than sodium nitrate for promoting growth of *D. salina*. Urea at the same concentration as potassium nitrate was found to be equally effective. But for an equal weight the amount of nitrogen available from urea is more than that from potassium nitrate. Ammonium salt was found least efficient. There are conflicting reports on the most assimilating form of inorganic nitrogen for *Dunaliella salina*. Different results have been reported regarding suitable inorganic nitrogen source (Borowitzka & Borowitzka 1988b, Giordano et al. 1994, Beardall & Giordano 2009). Nitrogen in the form of nitrate has been reported as a good source for culture of *D. salina* (Borowitzka & Borowitzka 1988b, Tafreshi & Shariati 2006). Thakur & Kumar (1999) reported that potassium nitrate supported better growth of *D. salina* than urea. Urea is reported to promote good growth but is not a preferred source in outdoor cultures due to release of ammonia (Borowitzka & Borowitzka 1990, Tafreshi & Shariati 2006). Urea can be used in well buffered laboratory cultures (Krol et al. 1997). There is also a report of favorable influence of nitrogen supplied in ammonium form to *D. salina* cultures (Beardall & Giordano 2009). Chlorophyll content and photosynthetic rate were found to double in *D. salina* cultures grown in medium with ammonium salts (Giordano et al.1994, Giordano & Bowes 1997). It has been reported that *Dunaliella* species can take up both nitrate and ammonium though the latter is used preferentially if both are available (Giordano et al. 1994). Cells acclimatized to growth on high ammonium concentrations usually showed similar division rates, but had a larger cell size, than their counterparts grown at high nitrate levels (Giordano & Bowes 1997). Ammonium salts have been reported to cause death of *D. salina* cells due to their acidifying effect (Borowitzka & Borowitzka 1988b). Ammonium in the form of NH₄NO₃ or (NH₄)₂CO₃ has been reported to have toxic effects on rapidly growing cells of *D. salina* (Borowitzka & Borowitzka 1988c). It has also been reported to effect carotenogenesis in *D. salina* (Krol et al. 1997). It appears that different strains of *D. salina* may prefer different forms of inorganic nitrogen.

In our experiments, trace elements except borate were found essential for growth of *D. salina*. Borowitzka (1990) reported that no work has been done to determine the requirement of trace elements in *D. salina* though they are added to the medium. Elimination of borate was found to boost growth of *D. salina*. Borate glass powder containing iron has been reported to be used for culture of *D. salina* (Yamaoka et al. 1997). Borate is absent in some media used for culture of *D. salina* (Subba Rao 2009). Borate was found to inhibit growth in *D. tertiolecta* (Ahmed et al. 1988). Thus requirement of borate is strain specific.

Vitamin mix was not found essential for growth of *D. salina*. In the survey of 326 microalgal species by Croft et al. (2006), a majority were found vitamin auxotrophic. It is believed that the vitamin autotrophy is confined to prokaryotes (Croft et al. 2005), and that all other species require vitamins in the medium in free form (Kurata 1986). It has been reported that *D. salina* does not require vitamins (Borowitzka
Mass culture units of *Dunaliella salina* & Borowitzka 1990). Media without vitamin mix are used for culture of *D. salina* (Subba Rao 2009). The composition of the media used by the industry is patent protected and it is not definitely known whether vitamin mix is added to the media in the industrial scale production of *Dunaliella*. At the concentrations added to Walne’s medium, the cost of the vitamin mix (B1, B12, and H) per 1000 L medium amounts to ~ 45 US$ (estimated from prevailing prices in Sigma Aldrich). Cells of several eukaryotic algae like *Tetraselmis suecica*, *Isochrysis galbana*, *Dunaliella tertiolecta* and *Chlorella stigmatophora* contain elevated concentrations of vitamins (Fabregas & Herrero 1990), perhaps acquired from the external medium. *Porphyridium purpureum*, a red microalgal species, and *Amphinidium operculatum*, a dinoflagellate is able to meet their vitamin requirement due to symbiosis with bacteria (Croft et al. 2006). In both these algae, the bacterium *Halomonas* spp. was implicated (Croft et al. 2006) as the unsuspected source of B12. The *D. salina* cultures in our study have also been found to harbour *Halomonas* spp. and the archaea *Halobacterium* (unpublished results).

The I3 isolate of *D. salina* was found to show optimal growth in a medium with 12.5 % NaCl. Isolates of *D. salina* are reported to differ in the optimal NaCl concentration required for growth. Different isolates of *D. salina* from Great Salt Lake were found to have NaCl optima of 10–15 % or even 19 % (Van Auken & McNulty 1973, Brock 1975).

Attempts of mixotrophic culture of *D. salina* have been reported. Suárez et al. (1999) reported good growth of *D. salina* in medium supplemented with sodium acetate or glycerol. In our study glycerol was not found suitable for growth of *D. salina*. Strains within a species were found to differ in response to different organic nutrients in our study with *Haematococcus pluvialis* and *Scenedesmus dimorphus*. In both *H. pluvialis* and *S. dimorphus* a strain that responded best to glucose did not show proper growth in medium with glycerol (unpublished results from our lab). Drokova & Dovhorouka (1966) reported enhanced carotene accumulation in *D. salina* cultures supplemented with carbon. Kim et al. (2012) found no success with any of the six carbohydrate sources (glucose, xylose, rhamnose, fructose, sucrose and galactose) they tested. Wan et al. (2011) reported good growth in mixotrophy with glucose. Mojaat et al. (2008) reported increase in carotene content when acetate or malonate was used in combination with Fe+2 in *D. salina* cultures. In our study both sodium acetate and malt extract when supplemented in mineral medium or sea water with 12.5 % NaCl were found to improve growth with the later being more effective. Sodium acetate is successfully used in the mass culture of *Haematococcus pluvialis* (Kaewpintong et al. 2007), a species closely related to *D. salina* and several other microalgae (Lee 2004, Perez-Garcia et al. 2011). Malt extract is a complex organic nutrient with complex carbohydrates and has been used as a source of organic carbon in *Tetraselmis suecica* (Azma et al. 2011). In our study a lower than optimal concentration of malt extract (when only malt extract is used in the optimized mineral medium) along with a higher than optimal concentration of yeast extract (when only yeast extract is used in the optimized mineral medium) was found to promote growth of *D. salina* without bacterial
growth. Yeast extract is not defined at a single element level and is a complex mixture with high carbon content and a mixture of nitrogen, vitamins, amino acids and trace elements (Grant & Pramer 1962) and has been suggested as a good nitrogen source (Chen et al. 2006, Azma et al. 2011). Peptone was used in the mixotrophic culture of *Tetraselmis suecica* (Huang et al. 2011) but in *D. salina* cultures, it was found to promote growth of coiled bacteria and the cultures became turbid.

The HPLC pigment profile of the I3 strain of *D. salina* matched with that reported earlier (Tafreshi & Shariati 2006). The carotene content (9.5 mg g$^{-1}$) in the dry mass that was observed in the autotrophic culture in our study is much higher than that reported (0.60 mg g$^{-1}$) (Cid et al. 1992). The carotene content (~56 mg L$^{-1}$) in mixotrophic culture and autotrophic culture seeded with culture grown mixotrophically (~27 mg L$^{-1}$) is much higher than values (8.1 to 15.1 mg L$^{-1}$) reported for commercial strains of *D. salina* grown autotrophically (García-González et al. 2003). The carotene content (112 pg cell$^{-1}$) in mixotrophically cultured *D. salina* is much higher than the reported value of 70 pg cell$^{-1}$ in medium supplemented with acetate and FeSO$_4$ (Gar- cía et al. 2007). When the seed culture was cultivated in mixotrophic mode and then mass cultured in autotrophic mode, biomass and β-carotene productivity was more than in a culture grown in an autotrophic mode throughout. The production cycle was reduced by 11 days with a three day saving of electricity consumed in laboratory (for air conditioning and lighting) during seed culture development which besides monetary gain also accrues environmental benefit of decreasing CO$_2$ release. Biomass and carotene content were even much higher in cultures grown only in mixotrophic mode. But cultures in medium with organic sources are reported to have problem of grazing (Benemann 2008) and therefore not suitable for intensive mass culture in race way ponds.

*D. salina* is cultivated in an extensive (open ponds/lagoons) or intensive (race way ponds) mode (Borowitzka & Borowitzka 1988a, Borowitzka 2010, Ben-Amotz 2004, Del Campo et al. 2007). Kitto & Rengunathan (2012) state that for intensive culture of *D. salina*, earthen unmixed pond culture is the only alternative to reduce the price of natural β-carotene. Feeding such ponds with mixotrophically generated seed culture and using the optimized mineral medium formulated in the present study would enhance productivity of *D. salina*.

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