**Development of sodium alginate encapsulation
of diatom concentrates as a nutrient delivery
system to enhance growth and survival of post-
larvae abalone (*Haliotis* spp.)**

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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		5 days visit in COMB	4 days in WAS conference	9 days of meetings
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Description of Cooperation: We worked together to develop an "off the shelf" and cost effective artificial diet for post larval (PL) abalone. Our studies were interdependent on each other in order to attain the main joint objectives. This cooperation was done through e-mail and telephone exchanges and the meetings noted above.

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The development of Sodium Alginate encapsulation of diatom concentrates as a nutrient delivery system to enhance growth and survival of post-larvae abalone (*Haliotis* spp.)

Abstract

The major bottlenecks in rearing the highly priced gastropod abalone (*Haliotis* spp.) are the slow growth rate and the high mortality during the first 8 to 12 weeks following metamorphosis and settling. The most likely reason for these problems is related to nutritional deficiencies in the diatom diet on which the post larvae (PL) feed almost exclusively in captivity. Higher survival and improved growth rate will reduce the considerable expense of hatchery-nursery residence time and therefore the production costs. BARD supported our research for one year only and the support was given to us in order to prove that "(1) Abalone PL feed on encapsulated diatoms, and (2) heterotrophic diatoms can be mass produced." In the course of this year we have developed a novel nutrient delivery system specifically designed to enhance growth and survival of post-larval abalone. This approach is based on the sodium-alginate encapsulation of heterotrophically grown diatoms or diatom extracts, including appetite-stimulating factors. Diatom species that attract the PL and promote the highest growth and survival have been identified. These were also tested by incorporating them (either intact cells or as cell extracts) into a sodium-alginate matrix while comparing the growth to that achieved when using diatoms (single sp. or as a mixture). A number of potential chemoattractants to act as appetite-stimulating factors for abalone PL have been tested. Preliminary results show that the incorporation of the amino acid methionine at a level of 10^{-3} M to the sodium alginate matrix leads to a marked enhancement of growth. The results of these studies provided basic knowledge on the growth of abalone and showed that it is possible to obtain, on a regular basis, survival rates exceeding 10% for this stage. Prior to this study the survival rates ranged between 2-4%, less than half of the values achieved today.

Several diatom species originated from the National Center for Mariculture (*Nitzschia laevis*, *Navicula lenzi*, *Amphora T3*, and *Navicula tennerima*) and *Cylindrotheca fusiformis* (2083, 2084, 2085, 2086 and 2087 UTEX strains, Austin TX) were tested for heterotrophic growth. Axenic colonies were initially obtained and following intensive selection cycles and mutagenesis treatments, *Amphora T3*, *Navicula tennerima* and *Cylindrotheca fusiformis* (2083 UTEX strain) were capable of growing under heterotrophic conditions and to sustain highly enriched mediums. A highly efficient selection procedure as well as cost effective matrix of media components were developed and optimized. Glucose was identified as the best carbon source for all diatom strains. Doubling times ranging from 20-40 h were observed, and stable heterotroph cultures at a densities range of 10^3 - 10^4 were achieved. Although current growth rates are not yet sufficient for full economical fermentation, we estimate that further selections and mutagenesis treatments cycles should result in much faster growing colonies suitable for a fermentor scale-up. As rightfully pointed out by one of the reviewers, "There would be no point in assessing the optimum levels of dietary inclusions into micro-capsules, if the post-larvae cannot be induced to consume those capsules in the first place." We believe that the results of the first year of research provide a foundation for the continuation of this research following the objectives put forth in the original proposal. Future work should concentrate on the optimization of incorporation of intact cells and cell extracts of the developed heterotrophic strains in the alginate matrix, as well as improving this delivery system by including liposomes and chemoattractants to ensure food consumption and enhanced growth.

Cooperation

The overall goal of the present study was to integrate the expertise of three research groups to develop an "off the shelf" and cost effective artificial diet that ensures good growth and survival for post larval (PL) abalone. This research encompasses a number of research milestones and the roles of the three research groups are interdependent on each other in order to attain the main objectives. The team in the US developed heterotrophic diatom production and the team in Israel has developed a nutrient delivery system for the diatoms while improving the PL's acceptance of them through the use of chemoattractants. Future work will rely and improve on the results stemming from this fruitful cooperation

Achievement

IOLR and ARO

Background

The major constraint in the rearing process of abalone is the slow growth from hatching to commercial size and the high mortality rate of PL during the first weeks following larval metamorphosis and settlement. It is likely that the main source of these problems is nutritionally based and therefore the focus must turn to diatom films, the principal food source for newly settled PL until individuals are weaned onto macroalgae. Researchers have observed that not all diatom species are digested and that many can pass through the gut whole and unbroken. Not surprisingly, post larval abalone consuming more digestible strains frequently demonstrated markedly better growth rates compared to PL grazing on more indigestible species. This suggests that diatom extracts may provide more accessible and consistent nutrition than mixed films of whole cell diatoms.

Chemical attractants play a dominant role in the feeding behavior of many aquatic organisms and their ability to locate food. It is therefore of major interest to identify the chemical cues involved in the induction of feeding in abalone. One artificial substrate that shows promise is the use of the Na-alginate encapsulation of diatom concentrates and extracts from strains that have demonstrated attraction and growth promoting characteristics. Our approach, which was tested successfully in preliminary studies, utilized a 1-2% Na-alginate solution to encapsulate diatom whole cell concentrates and extracts.

Research Objectives

1. Selection of diatom species that best attract the PL of the abalone species *Haliotis discus hannai*.
2. Testing the capability of these selected diatoms, intact cells or extracts, to attract abalone PL.
3. To evaluate PL growth performance on selected single diatom species. Testing it in a frozen or fresh form with and without sodium alginate encapsulation.

4. Comparison of growth in PL fed alginate alone or alginate encapsulating a heterotrophic algae (clone T, AquaGrow™ [heterotrophic cellular extract], and fresh diatom mixture).
5. Evaluation of PL growth on sodium alginate encapsulating selected chemo-attractants compared to sodium alginate alone and a mixture of fresh diatoms.

Experimental approach

The planned studies were conducted on the Japanese abalone *Haliotis discus hannai* from settlement size to 5 mm. This growth period extends from first feeding on diatom biofilms through weaning onto macroalgae. The abalone growth rate, survival, ingestion rate, digestion efficiency, and attraction to formulated Na-alginate encapsulated practical diets were determined.

Experimental set 1

Twenty-one benthic diatom species have been isolated and identified in our land based integrated system in Eilat (Shpigel et al., 1993) and natural abalone growing areas along the coast of southern California. Nine diatom species, selected from previous screening experiments, were tested for their capability to act as attractants the Japanese abalone. The selected species of diatoms were offered to ca 200 PL abalone simultaneously by placing them individually in small glass petri dishes (90 mm diameter) containing growth media treated with antibiotics and kept at 20 °C under 12L:12D light regime (Figure 1, 2). Each of the selected diatom species was tested in at least 10 replicates each, over a period of twenty-four hours. This method of utilizing behavioral spatial distribution in order to evaluate the attracting capability of diatoms has been used successfully for a number of organisms (Flores-Nava and Vera-Munoz 1999). The results showed that, of the nine species, two diatom isolates (*Nitzschia laevis* and *Navicula lenzi*) were found to most effectively attract the abalone larvae to settle (Table 1).

Experimental set 2

From the nine diatom isolates (Table 1), the best (*Nitzschia laevis*) and worst (*Amphora luceae*) performing isolates were tested in 10 replicates in three separate studies for attraction to abalone PL in two forms: as whole cell and as cell extract embedded in agar growth medium compared with agar growth medium as control. The results are presented in Figs. 3 and 4. They show that intact *Nitzschia laevis* markedly ($P < 0.05$) attracted the abalone more effectively than intact *Amphora luceae* and the agar control. On the other hand, the cell extract of *Amphora luceae* demonstrated the best attraction ($P < 0.05$) compared to the cell extract of *Nitzschia laevis*, suggesting that the former contains an effective attractant that is not expressed when the cell is intact.

Experimental set 3

The fact that abalone PL are attracted to certain species of diatoms doesn't necessarily mean that these algae promote good growth. In this experiment, growth and survival of PL abalone fed *Nitzschia laevis*,

Navicula lenzi and *Amphora* sp.(T.3), singularly and in combination, was tested. The results are presented in Fig. 5. They clearly show that a mixture of the three diatom species or T.3 and *N. lenzi* demonstrated the best PL growth. On the other hand, when *N. laevis* was tested alone, PL abalone exhibited the second best growth.

Experimental set 4

As different results were achieved with intact and broken diatom cells, in this set of experiments we tested the growth of PL abalone fed fresh or frozen mixture of diatoms (*Nitzschia*, *Navicula* and *Amphora*) embedded in alginate matrix. The results show that although alginate alone supported moderate growth, PL fed a mixture of fresh diatoms performed best (Figure 6). Moreover, frozen diatoms both free or embedded in the alginate matrix elicited a marked decline in growth, while a moderate growth reduction was observed when fresh diatoms were embedded in the alginate matrix. It can be concluded that the alginate is, indeed, consumed by PL. In order to achieve better growth, this matrix must include both attractant substances and other sources of nutrients to increase its suitability as an abalone artificial feed.

Experimental set 5

In the absence of sources of fresh heterotrophic diatoms, which at the time were in the final stages of development, other heterotrophic products were tested as additives to the alginate matrix. Alginate alone and alginate containing heterotrophic algal products (AquaGrow™ and Clone T) as well as selected diatoms were compared as growth and survival promoters in PL abalone. AquaGrow™ is a product produced in cooperation with Martek Biosciences (Columbia, Maryland, USA) and by co-principal investigators of this project at COMB (Center for Marine Biotechnology, Baltimore, Maryland, USA). This product is comprised of a mixture of phospholipid and lipid extracted meal from the heterotrophic algae *Cryptocodinium* sp. Clone T was also developed by Martek and COMB and consists of a spray dried whole cell preparation of a high lipid containing strain of this heterotrophic algae. Although the diatom treatment gave the best performance in PL abalone in terms of weight gain, feeding only alginate also contributed markedly to growth, supporting the results of the previous study (Experiment 4). However, when either of the heterotrophic products was included in the alginate matrix, the result was reduced growth, suggesting that these materials were not suitable for growing PL abalone. On the other hand, PL abalone feeding on these sources of heterotrophic algae demonstrated better survival than those feeding only on alginate and survived similarly to PL fed the selected diatoms (Figure 7). The conclusions from this trial reinforce the observations that Na-alginate offers base line nutrition for the PL and would be an appropriate delivery system for compounds that would improve its nutritional value. However, products derived from the heterotrophic algae *Cryptocodinium* sp.

performed poorly as growth promoters. The question remains if the addition of feed attractants to the alginate matrix would improve its acceptability and ingestion rate by the PL.

Experimental set 6

Evaluation of PL growth on sodium alginate encapsulating selected chemo-attractants compared to sodium alginate alone and a mixture of fresh diatoms. A number of potential chemoattractants at a level of 10^{-3} M were encapsulated in the Na-alginate. PL abalone were reared on the sodium alginate encapsulating selected chemoattractants and compared to sodium alginate alone (with no addition of attractants) and a mixture of fresh diatoms (with no Na-alginate) for a period of 42 days. Each of the diets was tested in five 20-L glass aquaria, each stocked with 150 post larval abalone. During the experimental period the total length, weight and survival were measured periodically. The results (Fig. 8) clearly show that the fresh diatom mixture supported the best PL growth. Among the Na-alginate diets, abalone PL growth was enhanced when the amino acid methionine (attractant # 2) was utilized as an attractant. As found in a previous experiment (experiment 4), the Na-alginate on its own with no addition of diatoms can support PL growth. Yet, when the PL abalone were given Na-alginate matrix containing the attractant, they exhibited better growth. This points towards the fact that the Na-alginate on its own has poor nutritional value, but the attractant incorporated in it led to enhanced consumption, thus enhancing the overall growth. Future work should explore the advantages of utilizing chemoattractants to enhance consumption and growth of abalone PL reared on artificial diets containing heterotrophic diatoms or other nutritional factors.

Figure 1: Experimental setup.

Figure 2: Cafeteria experimental setup.

Table 1: Attraction levels of postlarvae abalone to different species of diatoms in the “cafeteria” experiments.

Species	Attraction (%)
<u>Nitzschia laevis</u>	100
<i>Navicula lenzi</i>	85
<i>Amphora T3</i>	75
<i>Amphora coffaeformis</i>	62
<i>Nitzschia sp.</i>	58
<i>Navicula vimnoides</i>	37
<i>Navicula salinicola</i>	22
<i>Amphora tennerima</i>	20
<i>Amphora luceae</i>	0

Figure 3,4: Attraction levels of post larvae abalone to extracted intact diatom cells. Threedifferent abalone batches were of two species each+control.

Figure 5: Growth rate of post larvae abalone reared on single species diatom and mixed diatom diets.

Figure 6: Weight gain and survival rates of post larvae abalone reared on alginate mix and fresh diatoms embedded in aliginate matrix.

Figure 7: Weight gain and survival rates of post larvae abalone reared on alginate, AquaGrow TM and Clone T embedded in alginate matrix compared to fresh diatoms.

Figure 8: Weight gain and survival rates of post larvae abalone reared on alginate matrix and four potential attractants embedded in alginate and compared to diatom mixture.

COMB, USA

Background:

Recent studies in the national center for Mari-culture (NCM, Eilat, Israel) laboratories have indicated that larval abalone (*Haliotis* spp.) can actively absorb and ingest alginate matrix. Even more, supplementation of fresh diatom embedded in the alginate matrix to the culture medium was almost as equally attractive and nutritious as live algal feeds (NCM BARD report, experiment 4). Based on these results we have proposed to develop an inexpensive and highly nutritious source of heterotrophic algal derived food for the mass production of post larval abalone. The primary advantages of such heterotrophic species is that they can be cultured at much higher densities and at a significantly lower production costs than photoautotrophs. Autotrophic production of diatoms is expensive (\$50-400 per 1 kg dry weight) and inconsistent (Coutteau and Sorgeloos, 1992). On the other hand, the development of high density heterotrophic culture conditions to grow selected diatom species will be significantly more cost effective to produce for a practical compound diet. For example, maximum production efficiency of phototrophs is around 0.4 g/l/day whereas heterotrophic production can be over 10 times higher (over 5.8 g/l/day). In terms of energy investment, a production of 1 kg phototrophs may cost over \$11.22 in electricity (mostly as light) while under heterotrophic culture conditions, the cost of energy is only \$2.01 (cost of glucose) (This comparative information was obtained from Martek BioSci. Corp., Columbia, MD). Some diatoms are able to grow heterotrophically, in which organic nutrients are directly absorbed by the cell and provide all the nutrients necessary for the complete growth in the absence of light (a list of 35 such diatoms and references to experimental work is provided by Tuchman, 1996). It is believed that many diatom species out-compete other phytoplankton in the nature by having mechanisms to acquire energy and nutrients in highly turbid waters. This competitive success is believed contingent on the ability to supplement photosynthesis with heterotrophic uptake of organic compounds and to utilize two different pathways for assimilation of organic nitrogen under limited-light conditions. We hypothesized that relative to other micro algae many diatom species can be successfully culture by fermentation, because they are evolutionally suited to directly absorb organic nutrients. In fact, many diatoms can grow as rapidly heterotrophically as autotrophically, and some species grow faster. A few examples are the marine pennate diatoms, *Nitzschia alba*, and the centric diatom, *Cyclotella meneghiniana*, which grows heterotrophically in the dark if glucose concentrations between 5 mg/L and 10 g/L is provided (Hellebust, 1971; Hellebust and Lewin, 1977; Droop 1974).

A selected diatom strain must comply with a number of characteristics that would make it attractive for development as a heterotrophically grown abalone feed. The first character that we considered to be the most important was that the diatom must support high growth and survival rates of abalone larvae, conforming to NCM laboratory standards. We also considered several factors that most strongly affect the economics of heterotrophic production of the selected diatom strain. It must exhibit rapid and high-density growth, which is essential for low production cost; it must not require expensive fermentation nutrients; and the targeted species can grow at low salinities to reduce corrosion of the fermenting system. In this report we describe our attempts to identify diatom strains suitable for heterotrophic production as abalone feed. After screening several strains for heterotrophic growth, we chose 3 diatoms for further strain improvement; *Amphora T3*, *Navicula tennerima* and *Cylindrotheca fusiformis* 2083 UTEX strain.

Materials and methods

Diatom cultures: Agar plates inoculated with autotrophic cultures of *Navicula lenzi*, *Navicula tennerima*, *Nitzschia laevis* and *Amphora T3* were shipped from NCM laboratory to COMB. Five different strains of *Cylindrotheca fusiformis* (2083, 2084, 2085, 2086 and 2087 UTEX strains, Austin TX) were also screened for potential heterotrophic growth, because evidence exist that this diatom can grow heterotrophically (Darley and smiley 1976), and its silicon and glucose transporters were already characterized and sequenced (Hildebrand et al., 1998), and thus may be used as a control heterotroph reference in our studies with the NCM diatoms.

Culture medias: Instant ocean salt mix (Aquarium systems, Mentor OH) was used in place of seawater. Reagent grade chemical ingredients of the growth media were obtained from sigma (St. Louis MO). Screening for heterotrophic growth were performed in a variety of media ingredients combinations (Table 1) that based on inexpensive carbon sources (glucose, glycerol and a mixture of acetate lactate and glutamate) and complex nutrients (Table 2).

Axenic cultures: Initially all diatom strains were cultured in base medium containing an antibiotic mixture solution of 5000 units Penicillin, 5 mg streptomycin and 10 mg Neomycin per 100 ml. Antibiotic-treated diatoms were mixed (at near gelling temperature) with sterilized low gelling temperature agarose (0.7% SeaPlaque, FMC Bioproducts, Rockland, ME) and immediately poured into petri dishes in sterile hood (Stein, 1973). Inoculated agarose petri dishes were placed in the light at $20.5 \pm 1^\circ\text{C}$ incubator for 14 days and diatom colonies, separated from other diatom and bacterial colonies, were transferred to sterile base media tubes and allow to bloom. This procedure was repeated if bacteria or other contaminants were present.

Axenic diatom cultures were grown in sterile 16x120 mm screw-cap test tubes placed on a rotary platform shaker at 110 rpm in $20.5\pm 1^{\circ}\text{C}$ incubator (Innova 4230, New Brunswick Scientific, Edison, NJ). Axenic cultures were allowed to acclimate to mixotrophic conditions in base medium (BM) with added carbon source and complex nutrients (CN) nutrients in the light at 500-800 lux for 10-14 days before the start of heterotrophic growth experiments. Mixotrophic cultures were then used to inoculate the experimental test tubes. Each experiment consisted of an autotrophic control culture (base medium incubated in the light), a negative control (base medium incubated in the dark), and several combinations of base medium with added nutrients incubated in the dark. Evaluations of growth in each test tube were made by gross macroscopic and microscopic examination. Each culture was designated + or - for growth by comparing densities between heterotrophic culture tubes and light and negative controls.

Selective screening: Strain improvement for heterotrophic growth was performed routinely by inoculation of sterilized low gelling temperature agarose as outlined above and immediately poured into petri dishes in sterile hood. Inoculated agarose petri dishes were placed in the dark at $20.5\pm 1^{\circ}\text{C}$ incubator for 14 days and healthy diatom colonies, separated from other diatom and bacterial colonies, were transferred to sterile tubes containing base media with added sources of carbon and complex nutrients and allow to bloom in the dark (Fig.1). This procedure was repeated if bacteria or other contaminants were present. The selected diatom colonies were used again as the parent strain in each new cycle of selective screening.

Mutagenesis: Modification and further improvement of the selected diatom colonies for faster heterotrophic growth were achieved by subjecting sterile cultures of the selected diatom strains to 40 $\mu\text{g/ml}$ N-Nitrosoguanidine (NTG) for 20 min according to Baltz 1986. Diatom cultures were then centrifuged (1000gX15 min), supernatant decanted and diatoms mixed with sterilized low gelling temperature agarose as outlined above and immediately poured into petri dishes in sterile hood. Inoculated agarose petri dishes were placed in the dark at $20.5\pm 1^{\circ}\text{C}$ incubator for 14 days and healthy mutants diatom colonies, separated from other diatom and bacterial colonies, were transferred to sterile tubes containing base media with added sources of carbon and complex nutrients and allow to bloom in the dark (Fig.1). This procedure was repeated if bacteria or other contaminants were present. The selected diatom colonies were used again as the parent strain in a new mutagenesis cycle.

Table 1

Base Media (BM) for autotrophic culture, all concentrations are expressed per liter.

	BM1	BM2	NCM
Instant Ocean	60%	80%	100%
NaNO ₃	1.4 g	0.75 g	
KNO ₃			0.4 g
KCl	0.5 g		
NaH ₂ PO ₄ ·H ₂ O	120 mg	15 mg	50 mg
Na ₂ SiO ₃ ·5H ₂ O	200 mg	100 mg	420 mg
H ₃ BO ₃	30 mg	34 mg	
Na ₂ SeO ₃	6 mg	0.2 mg	
NaF	10 mg		
SrCl ₂ ·6H ₂ O	40 mg		
KBr	150 mg		
FeCl ₃ ·6H ₂ O	12.6 mg		10 mg
Fe ₂ SO ₄ ·7H ₂ O		20 mg	
Na ₂ EDTA	17.5 mg	5 mg	
MnCl ₂ ·4H ₂ O	720 µg	4.3 mg	360 µg
ZnCl ₂		0.3 mg	
ZnSO ₄ ·7H ₂ O	88 µg		44 µg
CoCl ₂ ·6H ₂ O	40 µg	0.13 mg	20 µg
NaMoO ₄ ·2H ₂ O	24 µg	25 µg	12 µg
CuSO ₄ ·5H ₂ O	40 µg	10 µg	20 µg
NiSO ₄ ·6H ₂ O		260 µg	
Vitamin B ₁₂	2.5 µg	300 µg	1 µg
Biotin	2.5 µg	300 µg	1 µg
Thiamine HCl	0.5 mg	6 mg	200 µg

BM1, 2 were formulated according to the recommendations of Martek biosciences Corp. (Columbia, MD). NCM, is base media formulation for diatom culture that is used at NCM laboratory.

Table 2

Additions to base medias (BM1,2) for heterotrophic testing

Carbon Source

Glucose or Glycerol or a mixture of

Acetate+Lactate+Glutamate 18 g

Complex Nutrient source (CN)

Bacto Tryptone 0.5 g/L

Yeast extract 0.5 g/L

NH₄Cl 25 mg/L

Urea 0.3 g/L

Bacto Tryptone and Yeast extract were obtained from Difco, Detroit, MI.

Figure 1. The process for selecting improved diatom strain with a faster heterotrophic growth.

NTG, N-Nitrosoguanidine

Results and Discussion

Based on data accumulated during this pilot project, we estimated the following diatom strains to have the best potential for heterotrophic culture, *Amphora T3*, *Navicula tennerima* and 3 strains of *Cylindrotheca fusiformis* (2083, 2086 and 2087 UTEX cultures). *Amphora T3* was described as an excellent feed for post larvae abalone, while a feed mix containing *Amphora T3* and *Nitzschia laevis* or *Navicula lenzi* permit the highest growth and survival rates. (Muki Shpigel, NCM, personal communications). Both *Nitzschia laevis* and *Navicula lenzi* however, failed to grow in the presence of highly enriched media with organic carbon and complex nutrients, indicating that these diatoms are probably not suitable for economical fermentor growth without some genetic engineering, either molecularly or by selection. Of interest was our difficulty to obtain sterile cultures of *Navicula lenzi*. This diatom grew very poorly in the presence of antibiotic, and agarose inoculations were consistently failed. We believe that symbiotic bacteria are essential for the development of *Navicula lenzi*, because they can assist with the adsorption of some nutrients. Evidence exists for other filamentous *Navicula* species (*Navicula confervacea*) that bacteria adhere to specific mucilage-free sites near areas of active nutrient uptake and release (Rosowski, 1992). It is well documented that healthy microalgae release dissolved organic carbon, which is utilized by bacteria (Jones and Cannon, 1986). For example,

vitamin B₁₂ deficient marine diatoms can have this requirement satisfied through association with B₁₂-producing bacteria (Haines and Guillard, 1974).

One of the most challenging tasks was to evaluate diatom growth by cell counting. Most diatoms grow in clustered colonies and adhere to the flask or vial wall (*especially Amphora T3*), and thus making sampling and individual cell counting impossible. Although in high density cultures or in a fermentor environment, where cultures must be agitated vigorously, this problem may be minimized, we employed several methods to separate the cells including treatment with a mixture of 1% CI and 0.1N NaOH, brisk sonication at low energy level, fluorescent count following staining with DAPI. However all methods failed to produce accurate and repeatable count estimates. Brisk sonication at low energy levels separated most of the cells in their intact form, but sampling was less accurate over time since many colonies were settled on the flask walls and sides. Therefore, we employed an evaluation method for the effect of the treatments that scored the number of colonies per unit volume and average colony size and health on a comparative basis (see figures 2-5).

Selection for fast growing heterotroph diatoms: We initially start the selection procedure only for diatoms that survive for 14 days in highly enriched media in the dark. Repeated selections of fast growing colonies in the dark employing the low gelling temperature agarose system (Fig.1) was very efficient selection method especially for *Amphora T3*, because this diatom grow in a form of very define and isolated colonies. Among the 5 *Cylindrotheca fusiformis* strains. Only 2086 UTEX strain grows in a form of define and isolated colonies while the rest of the strains grow in diffused form in the agarose, thus the selection system becomes less efficient. As a result of the repeated selections we were able to obtain faster growing colonies over time as can be seen in figures 2-5 as compared to non-selected colonies that grow poorly in the dark.

After about 10 selection cycles, diatoms were subjected to NTG mutagen treatment. *Amphora T3* responded poorly to this treatment and no further growth improvement were achieved. On the other hand, *Cylindrotheca fusiformis*, UTEX strains 2083 and 2086 and *Navicula tennerima* were very responsive, and healthy and dense blooms (at concentration of ca. 10⁴ cells/ml) obtained after 14 days in the dark (Fig 5.). Although doubling rates in the dark are still low (in the magnitude of 20-40 h, estimated by observing single cell multiplication rate on agar plates), we were very encouraged by the results of these selection procedures and strongly feel that over time of repeated selection much faster growth rates could be obtained especially for *Amphora T3* and *Cylindrotheca fusiformis*, UTEX strain 2083. *Navicula tennerima* seems to respond slower than the other two diatoms to this selection procedure, and so far we obtained only several colonies that grow in the dark. Nevertheless, we

recommend continuing the work with this diatom, because of its good value as abalone post larval feed and the fact that it responded to the selection treatments. Generally, doubling time of less than 10 h is required for efficient and economical fermentation production. For example, the doubling time in a selected strain of *Nitzschia alba* was 6.5 h, very similar to that of *Chlorella vulgaris*, which is translated to a production cost of ca. \$12/kg dry weight product (Gladue and Maxey 1994). Whereas production cost of ca. 20 h doubling time algae would range from \$160-600/kg dry weight (Kalk and Langlykke, 1986).

Culture Medias: NCN medium, which was specifically developed for culturing diatoms in the light, and BM1 were not sufficient to support good algal growth in the dark, while BM2 performed consistently better for almost all tested species (*Nitzschia laevis* grew equally well on both BM1&2 but completely failed to grow in the dark). Most of the strains that tested positive for heterotrophy were capable to grow with glucose as the carbon source and in the presence of complex nutrients. Addition of organic acids (a mixture of acetate+lactate+glutamate) as the carbon source resulted in poor cultures and high rate of bacterial contaminations. Figures 2 and 4 show the effect of carbon source with or without the addition of complex nutrients on colony growth of *Amphora T3* and *Cylindrotheca fusiformis* 2083 UTEX strain, respectively. They are clearly demonstrating that glucose is superior to glycerol as a source of carbon, as well as that yeast extract and tryptone are essential complex nutrients. Of interest was the variability within the *Cylindrotheca fusiformis* genus to grow in agar or in flask. *Cylindrotheca fusiformis* 2086 and 2087 UTEX strains grew very well in agar but didn't perform well when transferred to flask culture, while 2083 grow better in flasks containing BM2+Glu+CN. Overall, the BM2+Glu+CN matrix of media components is considered inexpensive and should permits low production costs.

Figure 2. The effect of carbon source (glucose or glycerol) and complex nutrients (CN) on growth of *Amphora T3* colonies. Several healthy and isolated colonies were selected from agar plates placed in the dark and transfer to 50 ml culture flasks containing sterile base media (BM2) with or without the addition of complex source of nutrients (CN) and glucose (Glu) or glycerol (Gly). Culture flasks were placed on a rotary platform shaker at 110 rpm in $20.5 \pm 1^\circ\text{C}$ incubator in the dark and allow to bloom. Pictures are showing a sample of average size colony (X4 ocular magnification).

Figure 3. Growth of *Cylandrotheca fusiformis* (2083, 2085, 2086 and 2087. UTEX strains) on agar plates in the dark. Antibiotic treated diatoms were mixed (at near gelling temperature) with sterilized low gelling temperature 0.7% agarose and immediately poured into petri dishes. Inoculated agarose petri dishes were allowed to bloom for 14 days in the dark at $20.5 \pm 1^\circ\text{C}$ incubator. Pictures are showing average size colony (X6 ocular magnification).

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