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Biotransformation of aromatic aldehydes by five species of marine microalgae

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Abstract

The biotransformation of a series of aromatic aldehydes such as benzaldehyde, salicy aldehyde, methoxybenzaldehydes and mono- and dichlorobenzaldehydes by five cultures of photosynthetic microalgae are reported. The microalgae, *Chlorella minutissima*, *Nannochloris atomus*, *Dunaliella parva*, *Porphyridium purpureum* and *Isochrysis galbana*, reduced most of the aldehydes to the corresponding primary alcohols. Substituted aromatic aldehydes were reduced with varying selectivity depending on the nature and position of the substituent.

Keywords: *Chlorella minutissima*; *Nannochloris atomus*; *Dunaliella parva*; *Porphyridium purpureum*; *Isochrysis galbana*; Microalgae; Marine biotechnology; Biotransformation; Aromatic aldehydes; Aromatic alcohols

1. Introduction

The biotransformation of organic substrates by fungal (Davies, Green, Kelly, & Roberts, 1990; Laskin, & Lechevalier, 1984) and plant cell cultures (Suga, & Hirata, 1990) has received considerable attention over the last number of years. Limited studies into the biotransformation of organic substrates by photosynthetic microalgae have been reported during this same period. These include the use of the unicellular green microalga *Dunaliella tertiolecta* in the biotransformation of a series of aldehydes (Noma, Akehi, Miki, & Asakawa, 1992) and the biotransformation of a series of aromatic aldehydes and ketones by photoheterotrophic cultures of *Euglena gracilis* (Noma, Okajima, Takahashi, & Asakawa, 1991; Noma, Takahashi, & Asakawa, 1991; Noma, & Asakawa, 1998). As part of an ongoing biotechnological study we report in this paper the results of a study into the ability of five species of marine microalgae to biotransform a series of aromatic aldehydes. The species under investigation include the green, non-motile, alga *Chlorella minutissima* (Chlorophyta), which has been used for many years as a research organism in the study of photosynthesis (Bold, & Wynne, 1978); *Nannochloris atomus* (Chlorophyta), a non-motile unicellular alga rich in eicasopentaenoic acid (EPA) and sterols; *Dunaliella parva* (Chlorophyta), a motile, unicellular chlorophyll-containing organism; *Porphyridium purpureum* (Rhodophyta), a non-motile, unicellular organism which is a potential source of the unsaturated fatty acids, EPA and arachidonic acids (Bold, & Wynne, 1978; Lee, 1989); *Isochrysis galbana* (Chrysophyta), a motile unicellular organism rich in the ω-3 fatty acids such as EPA and eicosahexanoic acid (Bold, & Wynne, 1978; Lee, 1989; Ewart, & Pruder, 1981).

2. Results and discussion

Initially the optimal growth patterns of the five microalgae were established. The growth of each test organism in static and shake cultures was compared over a four week growth period (Fig. 1, Table 1). For green microalgae *C. minutissima*, *N. atomus* and *D. parva* growth was paralleled by a linear absorbance at 440 nm using the method of Pirroeck, Baash, and Pohl

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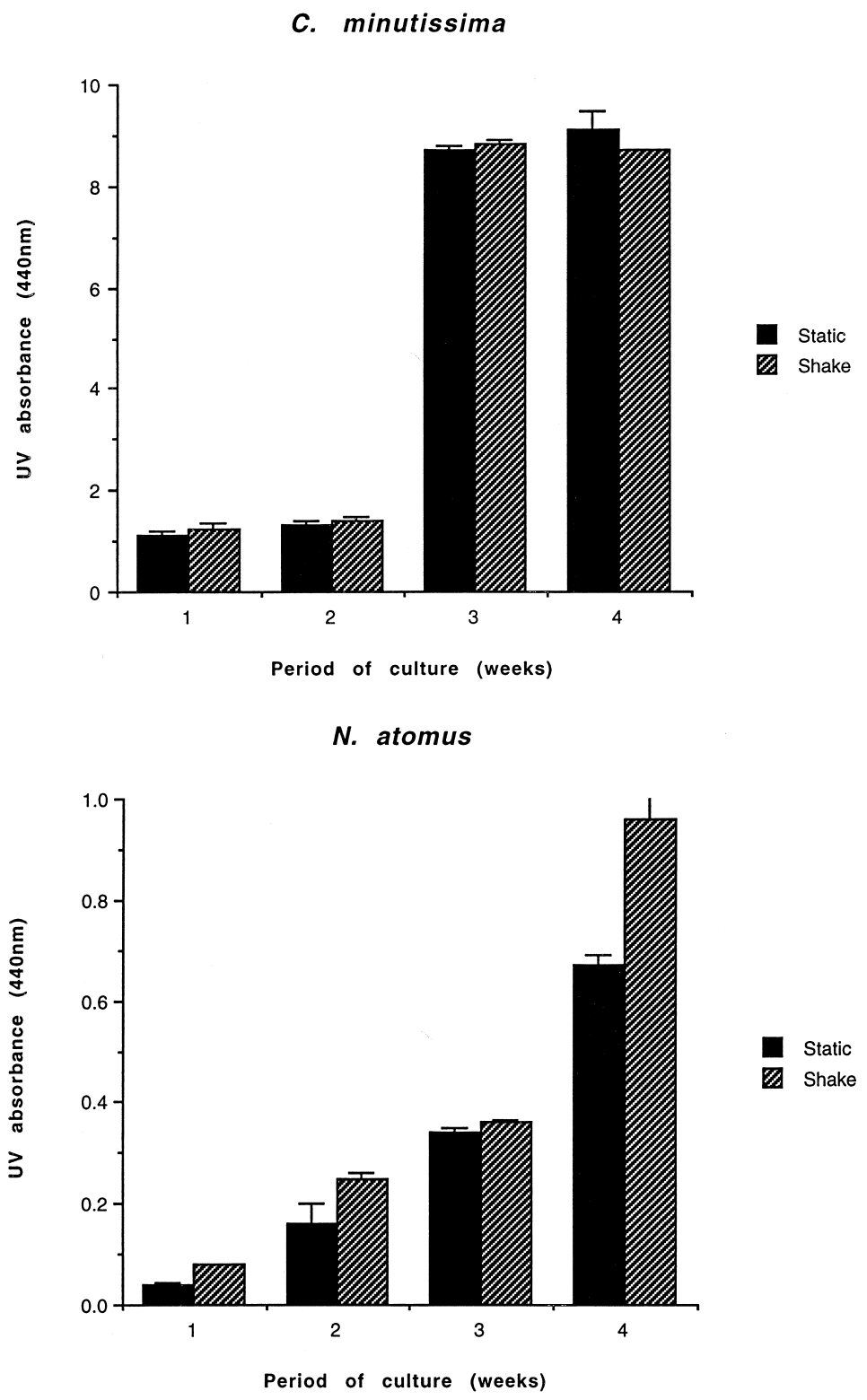

Fig. 1. Growth of test microalgae in shaken and static cultures measured over a four week growth period.

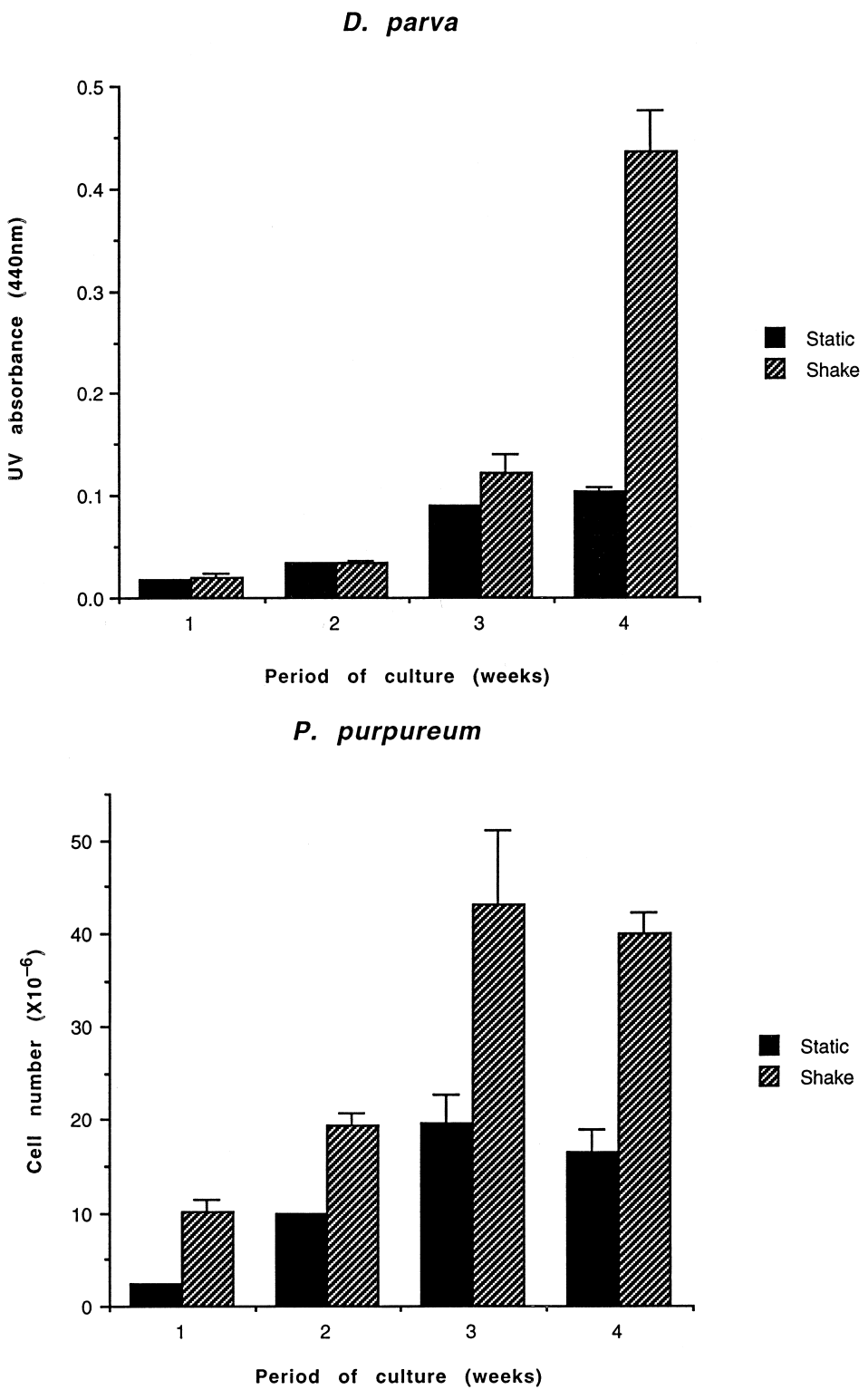

Fig. 1 (*continued*)

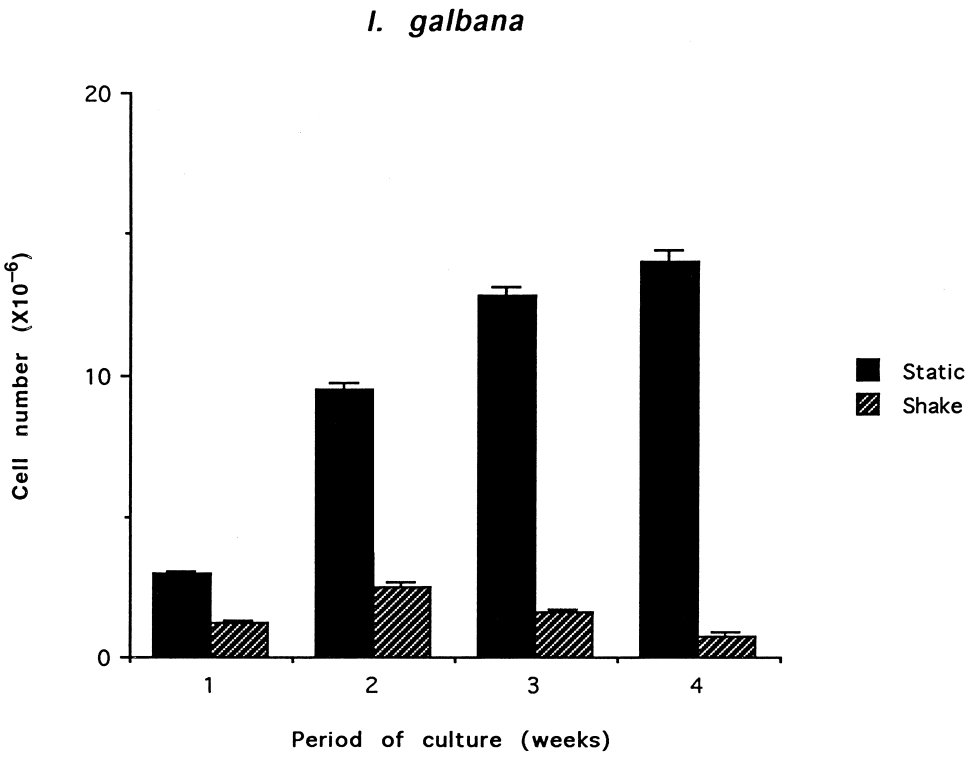

Fig. 1 (*continued*)

(1984). This method was not suited to measuring the growth of either *P. purpureum* or *I. galbana* where a haemocytometer was used (Kirsop, & Doyle, 1991). Results indicate that shake cultures of *N. atomus, D. parva* and *P. purpureum* led to an increase in growth relative to static cultures. Growth of *C. minutissima* increased dramatically in both shaken and static cultures between 2 and 3 weeks.

After four weeks, growth within static cultures was greater than within the corresponding shaken cultures. *D. parva* exhibited the greatest difference in growth on shaking, with this difference most dramatic after four weeks. Steady growth was observed in cultures of *N. atomus* over the four week period, after which time the best growth was observed in shaken cultures. The growth of *P. purpureum* in shake cultures was consistently higher than growth in corresponding static cultures. This observation may be attributed to increased uptake of nutrients on shaking. In static conditions cultures of *P. purpureum* also tended to grow as clumps of cells due to the presence of a thick sulphated polysaccharide which encapsulates the cells of all red microalgae (Fattarusso, & Piatelli, 1980; South, & Whittick, 1987). When cultures of *P. purpureum* are shaken the clumps of cells break up increasing the effective surface area available for nutrient uptake thereby increasing cell growth. In contrast with the other test organisms growth of *I. galbana* was favoured in static culture. Shaking cultures of *I. galbana* may interfere with normal motility and hence the organisms complex reproductive cycle (Bold, & Wynne, 1978; Lee, 1989; Borowitzka, 1988).

When static and shake patterns were compared for all five species (Fig. 1) it was decided that biotransformation studies for *I. galbana* would be carried out under static conditions whilst biotransformation by cultures of *C. minutissima*, *N. atomus*, *D. parva* and *P. purpureum* would be examined under shake conditions.

Table 1
Comparison in relative growth patterns between shaken and static cultures after four weeks growth

	Algal culture				
	C. minutissima	*N. atomus*	*D. parva*	*P. purpureum*	*I. galbana*
Static (%)	100	100	100	100	100
Shake (%)	96	150	419	245	5

The compounds chosen for study were a series of aromatic aldehydes including the parent aldehyde benzaldehyde (**1**), the 2-, 3- and 4-monochlorobenzaldehydes (**2**–**4**), the 2,3-, 2,4- and 3,4-dichlorobenzaldehydes (**5**–**7**), the 2-, 3- and 4-methoxybenzaldehydes (**8**–**10**) and vanillin (**11**). Using these compounds the relationship between structure and biotransformation ability of the test organisms was studied.

An initial experiment using various concentrations of organic substrates indicated that substrate toxicity increased with time and that after five days exposure to aromatic substrates all cultures showed a marked decrease in cell viability. In general substrates were most toxic to *I. galbana* and *P. purpureum*. The most susceptible green alga was *D. parva*. Substrate concentrations >200 ppm were too toxic so that biotransformation studies were carried out at concentrations of 100 ppm.

The biotransformation of aromatic aldehydes by the test microalgae is summarised in Table 2. Benzaldehyde (**1**) was readily reduced to benzyl alcohol by all species. This transformation was completed by *C. minutissima*, *N. atomus* and *P. purpureum* within a 24 h period. Cultures of *I. galbana* effected the biotransformation of **1** by the third day after inoculation while *D. parva* took four days to completely biotransform **1**. These results are in keeping with those observed by Noma et al. for the biotransformation of benzaldehyde by *D. tertiolecta* (Noma et al., 1992) and *E. gracilis* (Noma et al., 1991; Noma, & Asakawa, 1998). However for both species reduction was completed within 24 h. Although most of the substituted aldehydes were reduced to the corresponding primary alcohols not all of the chlorinated substrates were reduced by all of the microalgae.

After the five day period 2-chlorobenzaldehyde (**2**) was partially biotransformed by only two species, *C. minutissima* (9%) and *N. atomus* (1%). Selectivity in the transformation of **2** may be attributed to the proximity of the chlorine atom to the aldehyde group. Results are in contrast to those reported for *D. tertiolecta* which totally biotransformed **2** its corresponding alcohol within 24 h (Noma et al., 1992) and *Euglena* which carried out the same biotransformation but less efficiently (49%) (Noma et al., 1991; Noma, & Asakawa, 1998).

3-Chlorobenzaldehyde (**3**) was biotransformed to some degree by all the microalgal species. *P. purpureum* gave the best result with 99% of substrate biotransformed by the fourth day. *N. atomus* (83%) and *D. parva* (70%) reached their maxima after 5 days. In contrast cultures of *I. galbana* did not commence biotransformation of substrate until the third day after inoculation reaching a maximum biotransformation of 68% of **3** by the fifth day. *C. minutissima* was the least effective organism in the biotransformation of **3** with maximum yields of 10% being achieved after five days.

Maximum biotransformation of 4-chlorobenzaldehyde (**4**) occurred after five days for all microalgal species Table 2 with *D. parva* giving the highest yield (95%) of product after this time. However less than 1% of *p*-chlorobenzylalcohol was produced by *I. galbana*. We feel that this low level of transformation may be explained by the fact that **4** was more toxic to *I. galbana* than to other species.

A series of dichlorinated aldehydes were also incubated with the five algal cultures Table 2. Less than 1% of 2,3-dichlorobenzaldehyde (**5**) was biotransformed to the corresponding alcohol by all species. Results are in keeping with the low levels of biotransformation observed for 2-chlorobenzaldehyde (**2**) by all species. In contrast, 2,4-dichlorobenzaldehyde (**6**) was biotransformed to a greater extent than **5** Table 2. Maximum reduction of **6** was carried out by *D. parva* (12%) and *N. atomus* (7%).

Only partial reduction of 3,4-dichlorobenzaldehyde (**7**) was also observed within the test period. Maximum conversion was carried out by *D. parva* (27%) and *P. purpureum* (22%). *I. galbana* metabolised less than 1% of substrate within the five day period Table 2. When the biotransformation of **5**, **6** and **7** is compared with that of 2-chlorobenzylaldehyde (**2**), 3-chlorobenzylaldehyde (**3**) and 4-chlorobenzaldehyde (**4**), a significant

Table 2
Relative % biotransformation of aromatic aldehydes (**1**–**11**) by all algal species after five days (* after nine days)

Culture	Substrate										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
C. minutissima	100	9	10	80	< 1	1	6	100	97	100	0
N. atomus	100	1	83	89	< 1	7	3	100	100	97	0
D. parva	100	0	70	95	< 1	12	27	94	98	97	0
P. purpureum	100	0	99	76	< 1	4	22	100	58	100	0
I. galbana	100	0	68	1	< 1	1	1	100	96	100	0
D. Tertiolacta (Noma et al., 1992)	100	100	100	100	–	–	–	100	100	100	25*
E. Gracilis (Noma et al., 1991; Noma, & Asakawa, 1998)	100	100	100	100	100	100	100	100	100	100	100

decrease in the efficiency of transformation of the dichlorinated substrates is observed.

The biotransformation of a series of monomethoxylated aldehydes was also investigated in this study. The reduction of 2-methoxybenzaldehyde (**8**) was completed within five days by all of the test microalgae with the exception of *D. parva* which achieved a maximum biotransformation of (94%) within this time frame. *I. galbana* had completed the total biotransformation of this substrate after three days. In contrast *D. tertiolecta* and *E. gracilis* totally biotransformed this substrate within 24 h (Noma et al., 1992; Noma et al., 1991; Noma, Takahashi, & Asakawa, 1991; Noma, & Asakawa, 1998).

A more selective reduction of 3-methoxybenzaldehyde (**9**) was catalysed by *P. purpureum* with only 58% of substrate being transformed in the five day period. In fact this maximum was observed after four days and did not alter after this time. The other organisms reduced the substrate as effectively (>95%) as the literature examples.

Almost total biotransformation of 4-methoxy benzaldehyde (**10**) occurred within the five day period Table 2. The biotransformation was more rapid than that of **8** and **9** with 75% being transformed by *C. minutissima*, *I. galbana* and *P. purpureum* within the first three days. *N. atomus* and *D. parva* reached this level on day four. The results for the monomethoxylated substrates contrast with those published for *D. tertiolecta* which gives the same primary alcohols when incubated with **8**, **9** and **10** once again the biotransformations are total and occur within a 24 h period.

The biotransformation of vanillin (4-hydroxy-3-methoxybenzaldehyde, **11**) was also investigated in this study but it was not metabolised by any of the algal species under investigation. This may be due to the deactivating effect of the 4-hydroxyl group previously reported by Noma et al. (1992) who have observed that substrates with hydroxy and nitro substituents at the 2- and 4-positions were difficult to transform or were not transformed at all. Some biotransformation of **11** has been reported for *D. tertiolecta*, where the only product observed was the primary alcohol. However this biotransformation was less efficient than for the other substrates with a maximum biotransformation (25%) being observed after nine days (Noma et al., 1992).

All the algal species investigated demonstrate the reduction of the aromatic aldehydes to their corresponding alcohols to some extent. Substituted aromatic aldehydes were reduced with varying efficiency depending on the nature and position of the substituent. The chlorinated benzaldehydes were reduced less efficiently than their methoxylated counterparts. Of the dichlorinated substrates 3,4-dichlorobenzaldehyde (**7**) was biotransformed to the greatest extent, however it was reduced to a lesser extent than the 3- and 4-monochlorinated counterparts. Methoxyaldehydes were all reduced with the exception of vanillin a result which is consistent with literature reports (Noma et al., 1992).

Of the organisms under investigation *D. parva* exhibited the greatest ability to biotransform mono- and dichlorobenzaldehydes while *N. atomus* was best at biotransforming methoxyaldehydes. The organisms under investigation in this study exhibit greater selectivity in the reduction of aromatic aldehydes than *D. tertiolecta* or *E. gracilis*. The ability of the test organisms to biotransform substrate appears to be dependent on the nature and position of the substituent.

The present method of study is useful and the workup is easy, however a long interval is required for reaction to occur. The ability of the test organisms to selectively biotransform a range of natural and synthetic products is in progress.

3. Experimental

3.1. General experimental procedures

Tlc was carried out on plastic sheets pre-coated (0.2 mm) with Si gel $60F_{254}$ (Merck). GC analyses were carried out using a Perkin Elmer Sigma-3 GC, FID glass column (2 m; 1.75 mm i.d.) packed with 15% Carbowax 20 M on Gas Chrom. WAW (80–100 mesh), operated at various temperatures. N_2 flow rate 30 ml/min. For dichlorinated benzaldehydes GC analyses were carried out using a Perkin Elmer Gas Chromatograph 8700 fitted with a stainless steel-glass lined column: 1×6 ft GLT:$\frac{1}{8}$ in; mm i.d., packed with Carbowax 20M (10%) on Chromosorb WAW (100–120 mesh). Injector temp: 220°C, oven temp.: 195°C, detector temp: 195°C. N_2 flow rate 30 ml/min.

3.2. Algal cultures

The algae used in this experiment were *Chlorella minutissima* Fott and Novakova[a]; *Nannochloris atomus* Butcher[a]; *Dunaliella parva* Lerche[b] *Isochrysis galbana* (*T-Isochrysis*)[a]; *Porphyridium purpureum* [b] ([a]Regional Technical College, Galway, Ireland, [b]Carolina Biological Supply Company, USA). The stock cultures were maintained in an artificial sea water (ASW) culture medium and cultured for 30 days (Mockler, 1992). The algae were grown at a constant temperature of 25°C, and illuminated by cool white fluorescent tubes kept on a six/eighteen hour dark/light cycle at light intensity of 20 W m^{-2}. At subculture 1:10 or 1:5 dilutions were made of algal broth into sufficient fresh ASW to give a 40% flask fill. All procedures were carried out using strict aseptic protocol. All determinations were carried out in triplicate. Algal dry

weights were determined from 25 ml aliquots of cultures filtered through a 1.2 μm membrane filter and dried in a fan assisted oven $<40^{\circ}C$. Growth of the algae was assessed on day 30 of the growth cycle. Growth of all green algae was monitored by measuring the absorbance of whole cells at 440 nm. Growth of cultures of *P. purpureum* and *I. galbana* was determined using a haemocytometer with Neubauer rulings in a 1 mm^3 chamber. Each algal type (1:5 dilution) was set up in conical flasks (six, 100 ml) (8 ml of algal broth in 32 ml ASW). Growth under standard static and shaking cultures was measured after 7, 14, 21 and 28 days. Shaking cultures were placed on an orbital shaker set at 90 rpm.

3.3. Toxicity measurement

Each algal type (1:5 dilution) was set up in 10 ml pyrex test tubes (1 ml algal broth in 4 ml ASW). Growth under standard static conditions was allowed for seven days. Substrates were dissolved in methanol and 1 ml of this solution was added to the algae to bring final substrate concentrations to 100 ppm. In addition two types of control were used. Control 1: algae + ASW only; and control 2: algae + ASW + 1 ml methanol (i.e. substrate free). All concentrations and controls were set up in triplicate. To monitor toxicity, aliquots (0.5 ml) were removed aseptically from the culture tubes at intervals of 24, 48, 72, 96 and 120 h. Survival of all the green algae was monitored by measuring the absorbance of whole cells at 440 nm. Survival of *Isochrysis* and *Porphyridium* was determined by cell count using a haemocytometer.

3.4. Biotransformations

The culture conditions are as outlined above. Dilutions of microalgae (1:5) were made in conical flasks (250 ml) to provide a 40% flask fill (i.e. 20 ml algal broth into 80 ml ASW). Algae were first allowed to grow statically for seven days at which stage a solution of substrate in methanol (1 ml) was added. Aliquots (10 ml) were taken from culture flasks in triplicate at intervals of 24, 48, 72, 96 and 120 h. These were extracted with equal volumes of distilled Et_2O. The ether layer was separated, dried over Na_2SO_4, filtered and evaporated to dryness in vacuo. The residue was re-dissolved in Et_2O (1 ml) and analysed under standard GC conditions. The three replicates for a given data point of the time course experiment for each alga were combined. The components of the diethylether extracts were separated either by column chromatography on silica gel (Si gel $60F_{254}$, E. Merck, Darmstadt) or by prep. tlc. Products were visualised by UV light (254 nm, as dark quenching bands) or using 1% vanillin in conc. H_2SO_4 (100°C for 5 min) or freshly prepared iodoplatinate spray.

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