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Co-Immobilization of Symbiotic Green Algae and *Saccharomyces unispora*

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Abstract

The effects of co-cultivation on the proliferation of the heterotrophic yeast *Saccharomyces unispora* and the symbiotic green algae (*Chlorella sorokiniana* 211.40c and *Chlorella saccharophyla* 3.80) were studied in a Ca-alginate gel matrix. The results suggest that due to their assimilative and transport processes the symbiotic green algae provided conditions for significantly increased growth of this yeast. The beneficial effect of co-cultivation with *C. sorokiniana* 211.40c is indicated by the higher cell counts. In parallel with the higher total algal and yeast cell concentrations, the spatial distribution of the cells inside the bead changed markedly. The detection of yeast-specific compounds such as butyric acid in the medium suggests the possibility of linkage between the metabolic pathways of the symbiotic algae and the yeast.

Keywords: *Chlorella*, symbiosis, co-immobilization, co-cultivation, yeast

1. Introduction

Studies on symbiosis involving unicellular algae such as *Chlorella* and their host organisms have been reviewed extensively (Muscatine, 1967; Jolley and Smith, 1978; Fischer et al., 1989). The individual characteristics of the symbiotic partners, for example the regulation of the proliferation of the

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photobiont by the host organism or the sugar excretion of photobionts have been examined (McAuley, 1985; Bossert and Dunn, 1986). The sugar excretion of cultivated symbiotic *Chlorella* strains is a well-defined parameter, affected by the environmental conditions and regulated by the physiological condition of the host organism (Mews and Smith, 1982; Wang and Douglas, 1998). The glucose release by *Chlorella sorokiniana* 211.40c (photobiont of *Spongilla fluviatilis*) has been reported to have a pH optimum in the acidic range (Fischer et al., 1989). The maltose excretion of the photobiont from *Hydra viridis* displays a similar pH profile (Mews and Smith, 1982). Although these studies have touched upon the aspects of sugar excretion and its conditions, there have been few studies of the utilization of the synthesised and released sugars (Brechignac, 1990).

Another important field of symbiosis research is the examination of the relationship between photobionts and host animals, emphasizing the animal aspect of the relationship. Several characteristics of natural symbiosis, such as the host specificity and the ability of green algae to form stable symbioses, have been reconstructed by re-establishing symbiosis, using the original photobionts or non-symbiotic algae (Görtz, 1982; Bossert and Dunn, 1986).

The aim of this study was to assemble a system for the cultivation of a heterotrophic organism, based on the natural processes of the algae. Assimilative and transfer processes of symbiotic algae were expected to ensure the necessary organic sources for the partner organisms in organic C- and N-free medium. Evidence for the functionality of the co-cultivated system would be continuous growth and detectable products of the heterotrophic partner.

In order to conform with the pH-dependence of sugar release by the *Chlorella* algae strains used, a heterotrophic partner-microorganism preferring acidic conditions, a yeast strain (*Saccharomyces unispora* isolated from whey), was selected.

With a view to mimicking natural physiological connections as closely as possible, the gel-entrapping method was applied to ensure the close proximity of the photobionts and their heterotrophic partner.

2. Materials and Methods

Microorganisms and growth conditions

C. sorokiniana 211.40c (photobiont of *Spongilla fluviatilis*) and *C. saccharophyla* 3.80 (phycobiont of *Trapelia cordata*) were obtained from Sammlung für Algenkulturen Göttingen. The algae were cultivated under axenic conditions in a liquid medium containing the following components: 200 mg l^{-1} KNO_3, 20 mg l^{-1} $MgSO_4.7H_2O$, 20 mg l^{-1} K_2HPO_4, and 1000 mg l^{-1} 3-[N-

morpholio]propanesulfonic acid (MOPS), the pH being adjusted with phosphoric acid (25% w/w) to 4.2. FeSO4 and trace elements were as given by Kuhl (1962). Cells were harvested after 6–7 days at cell densities 0.8–1.2 OD (660 nm) and resuspended in the previously described medium. This medium was used in the experiments described (SAG).

Saccharomyces unispora isolated from whey was cultivated in liquid medium, using universal medium for yeast (DSMZ-YM: yeast extract 3 g l^{-1}, malt extract 3 g l^{-1}, peptone 5 g l^{-1}, and glucose 10 g l^{-1}) for 24 h in 100 ml Erlenmeyer flasks on a rotary shaker, with mild (50 rpm) mixing at room temperature.

Immobilization: The algae and yeast were immobilized in a Ca-alginate matrix (Sigma HV from *Macrocystis pyrifera*) using a 2% initial Na-alginate concentration according to the following protocol. Cells were collected by centrifugation at 5,000 rpm (Sorval RT 6000D) for 10 min, when the culture had reached a cell density of 10^7–10^8 ml^{-1}. The pellet was suspended in fresh SAG medium and 1 ml of cultures of *C. sorokiniana* 211.40c or *C. saccharophyla* 3.80 containing 2.0–4.0 × 10^7 cells were mixed with 9 ml of Na-alginate solution. The algal suspension obtained was introduced dropwise into 0.1 M $CaCl_2$ solution with a plastic syringe. Due to the size of the applied plastic tips (inside diameter 0.5 mm), the diameter of the beads was 2.9–3.0 mm. The beads formed were left for 6 h at 25°C and then washed in fresh SAG medium.

In the case of alga – yeast co-immobilization, the same method was used, but with the addition of 1 ml of *Saccharomyces* culture containing 10^7 cells suspended in fresh SAG medium.

Culture conditions for co-immobilized microorganisms or organisms alone

Three ml (60–70 beads) of co-immobilised culture or *C. sorokiniana* 211.40c, *C. saccharophyla* 3.80 or *S. unispora* alone was cultivated in 15 ml of SAG medium in batch cultures. Batch cultures were incubated in Erlenmeyer flasks at 24°C and 100 rpm in a Gallenkamp illuminator for 14–16 days.

Location of microorganisms inside the bead

The total cell density and the partial distribution in the Ca-alginate beads were determined by microscopic counting after gradual dissolution of the Ca-alginate matrix, using the method described by Boross et al. (1990).

Determination of organic acid and glucose

Organic acids (butyric acid and lactic acid) and ethanol fermented by

Saccharomyces and glucose released by *Chlorella* strains were separated and determined on a Gynkotek isocratic HPLC system using a SARASEP Polymer CAR-H column, a Gynkotek UVD 160 detector and a Shodex RI-71 refractive index detector. The eluent was 0.01 N H_2SO_4 and the flow rate was 0.9 ml min^{-1} at 50°C.

Statistical analysis

Batch cultures were prepared in triplicate, a single flask serving as one replicate, and each experiment was repeated three times. For cell number determination in the entrapping material, six beads were taken at random for a count of the total cell concentration in each culture. In order to determine the location of the cells within the beads, three beads were taken at random from each culture and the test was repeated three times. The mean values of the index were compared with those for the control, using the Student t-test.

To decide the significance of the results of the cell growth curves, a nonlinear fit (Boltzmann equation) was applied. Parameters were compared with the control data by using the Student t-test.

3. Results

Total cell number analysis of microorganism cultures within alginate beads

Marked differences were observed after 14–16 days of incubation for the growth curves of algae co-cultivated with yeast and without yeast. While the *C. sorokiniana* 211.40c concentration reached a level of 7.5–8 × 10^7 cells/bead in the presence of *Saccharomyces unispora*, the cell concentration obtained without the yeast was only 4–5.2 × 10^7 cells/bead. However, co-cultivation had no effect on the proliferation of the other algal strain, *C. saccharophyla* 3.80: approximately the same total cell concentrations were measured in both cases (1.6-1.8 × 10^7 cells/bead) (Fig. 1).

During the incubation period, the algal cells were counted, and also the level of the yeast cells was observed. The cell concentration of yeast immobilized without algae decreased continuously. When co-cultivated with the algae, the yeast grew vigorously. The growth rates differed with the different algal strains. By the end of the incubation period, significantly more cells developed when *C. sorokiniana* 211.40c was used (8.2 × 10^6 yeast cells/bead) than for the other strain, *C. saccharophyla* (4.4 × 10^6 yeast cells/bead) (Fig. 1).

Exact determination of the effects of co-cultivation on the growth of the algae and yeast demanded statistical analysis of the growth curves. For evaluation of these curves, the slope of the log phase of the curves (μ) was

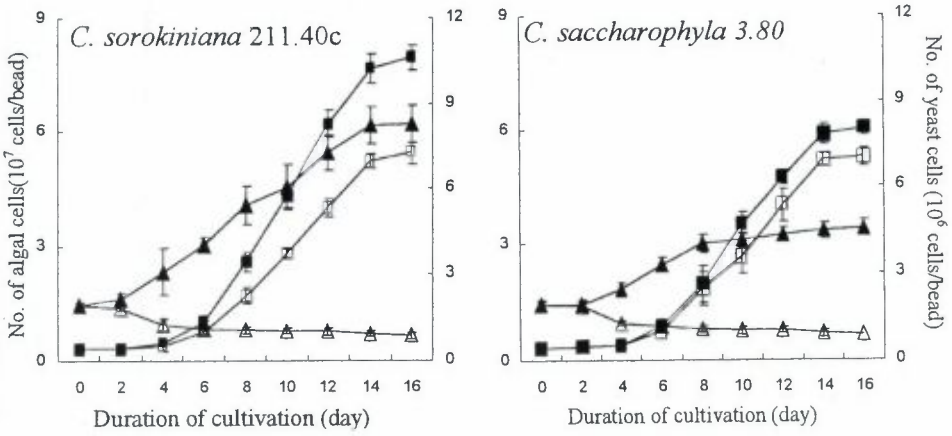

Figure 1. Effect of co-cultivation on proliferation of immobilized *C. sorokiniana* 211.40c or *C. saccharophyla* 3.80 and *S. unispora*. Growth of symbiotic algal strains immobilized alone (❑) or with *S. unispora* (■); growth kinetics of *S. unispora* alone (Δ) or with symbiotic algal strains (▲). Bars represent the standard error (SE). When the SE bar is absent, the SE is smaller than the point.

Table 1. Results and parameters of the chosen statistical Boltzmann equation

Algal strain	α	β	μ	R2
C211.40c	2.47	58.50	2.08	0.997
Y C211.40c	2.2	83.73	1.93	0.998
C3.80	2.69	57.16	2.05	0.994
Y C3.80	2.54	62.69	1.85	0.998

C211.40c: *C. sorokiniana* 211.40c immobilized alone; Y C211.40c: *C. sorokiniana* 211.40c immobilized with *S. unispora*; C3.80: *C. saccharophyla* 3.80 immobilized alone; Y C3.80: *C. saccharophyla* immobilized with *S. unispora*; α: initial cell concentration ($\times 10^6$ cells $bead^{-1}$); β: cell concentration of the stationary state ($\times 10^6$ cells $bead^{-1}$); μ: slope of the log phase; R^2: goodness of fit.

defined by using the Boltzmann equation; other parameters were measured e.g. the initial (α) and final (β) cell counts (Table 1).

In order to determine the significance of differences of growth curves, the two-tailed P values were determined with the Student t-test between the coherent parameters.

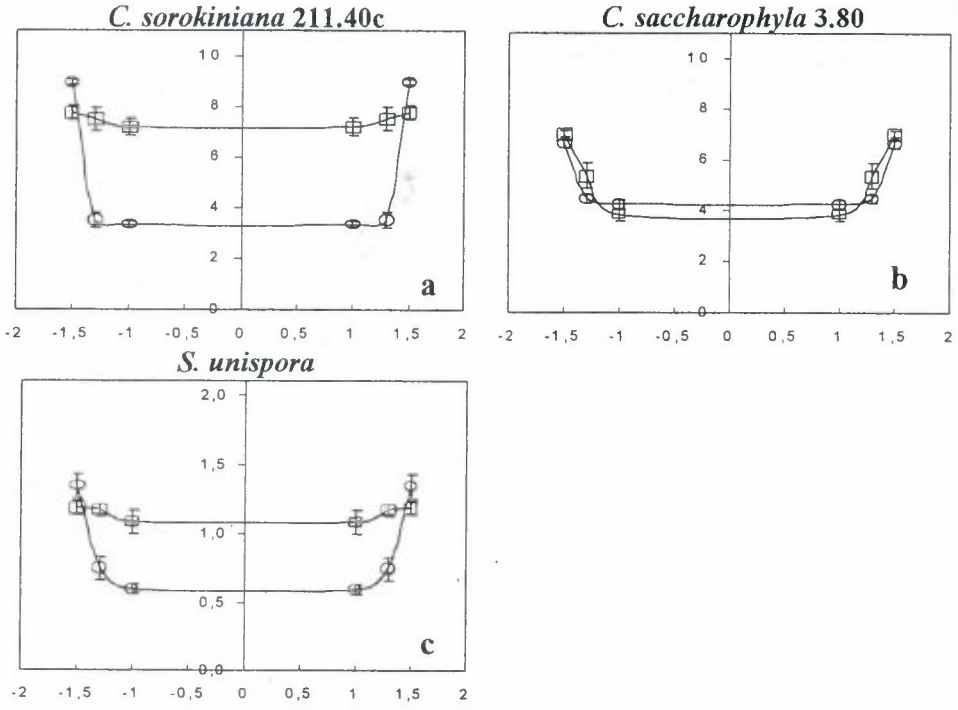

Figure 2. a) and b) Spatial distribution of symbiotic green algae immobilized alone (o) or with *S. unispora* (❑). c) Spatial distribution of yeast *S. unispora* immobilized with *C. saccharophyla* 3.80 (o) or with *C. sorokiniana* 211.40c (❑). The origin represents the center of beads, and the axis x shows distances from the center in mm. Bars represent the standard error (SE). When the SE bar is absent, the SE is smaller than the point.

Significant differences in the cell counts of the stationary states were found when *C. sorokiniana* 211.40c was co-cultivated with the yeast or without it ($P<0.0001$). Although the total cell concentration attained was higher, the kinetics of the growth rate did not differ from those for the cultures immobilized alone ($P=0.579$).

Spatial distribution of Chlorella and yeast cells immobilized alone or co-immobilized

When immobilized alone both *Chlorella* strains preferred to grow in the periphery of the beads. The picture of the distribution changed significantly when *C. sorokiniana* 211.40c was co-immobilized with the *Saccharomyces* strain. The number of algal cells in the interior of the beads increased, and the

distribution became more uniform, approaching the initial, homogeneous distribution seen immediately after immobilization.

When *C. saccharophyla* 3.80 was co-immobilized with *Saccharomyces unispora*, its distribution did not change significantly (Figs. 2a and b).

The spatial distribution of the yeast cells showed patterns similar to the algal localization in the entrapping material. When *C. sorokiniana* 211.40c was used, the yeast distribution was more homogeneous and the cells preferred the outside layer of beads as compared with cultures containing *C. saccharophyla* 3.80 (Fig. 2c).

Products of Saccharomyces in the co-immobilized system

Analysis of the medium of co-cultured symbiont *Chlorella* algae and *Saccharomyces*, revealed the presence of butyric acid, a yeast product. Other expected products (lactic acid and ethanol) were detectable only in traces, corresponding to the kinetics of the appearance of butyric acid in the medium (data not shown). With *C. sorokiniana* 211.40c, the levels of fermentation products were higher than in the case of *C. saccharophyla* 3.80, where the concentration of ethanol was below the detection level. These values closely follow the levels of glucose released by algae immobilized alone; further, fermentation products indicative of yeast growth appeared only in the presence of either symbiotic algae or an external organic carbon source such as glucose (Table 2).

4. Discussion

Co-cultivation and co-immobilization of symbiotic *Chlorella* strains with *Saccharomyces unispora* resulted in enhanced yeast proliferation, whereas the cell concentrations of yeast cultures free of algae decreased. Although yeast proliferation was stimulated by both algal strains applied, there were differences between the highest yeast concentrations attained in the entrapping material. Application of *C. sorokiniana* 211.40c strain resulted in an almost two-fold increase in maximal cell concentration of *Saccharomyces unispora* as compared with the case of co-cultivation with *C. saccharophyla* 3.80.

The possibility of a linkage between the metabolic processes of *Chlorella* strains and *Saccharomyces unispora* is supported by the demonstration of yeast-specific products such as butyric acid and other organic compounds, since the appearance of these compounds is indicative of the utilization by the yeast of the glucose released by the alga. This suggestion is supported by the observation that the level of butyric acid produced by *Saccharomyces*

Table 2. Compounds in the medium of immobilized and co-immobilized cultures (mg compounds ml^{-1} beads). Samples were taken on day 12 of the incubation and values were determined by HPLC

	Algal strain	Glucose in medium	Butyric acid in medium	Other compounds
S. unispora	*C. sorokiniana* 211.40c	0	15±0.9	Lactic acid: <1 Ethanol: <1
	C. sorokiniana 211.40c	60±5	0	0
S. unispora	*C. saccharophyla* 3.80	0	<4	Lactic acid: <2 Ethanol: n.d.
	C. saccharophyla 3.80	5±0.5	0	0
S. unispora (+100 mg l^{-1} external glucose)		0	6±0.1	Lactic acid: 5±0.4 Ethanol: 1±0.1
S. unispora (without organic carbon source)		0	0	0

correlates with the level of glucose released by the symbiotic algal strains (Table 2).

On the other hand, butyric acid appeared in the presence of either alga or an external organic carbon source (glucose) and the glucose released by the algae co-cultivated with the yeast was not detected during the period of co-cultivation (Table 2).

Since both yeast proliferation and the production of organic compounds by yeast were more intensive in the presence of *C. sorokiniana* 211.40c, the physiological aspects (rate and amount of glucose release) of the algal strain used seem to play a central role in the support of its heterotrophic partner.

Examination of the effects of co-cultivation on the natural symbiotic algal partner showed that the growth rate of *Chlorella sorokiniana* 211.40c immobilized with *Saccharomyces unispora* increased. Analysis of the cell growth curves by statistical methods revealed that co-cultivation of *Chlorella sorokiniana* 211.40c with *Saccharomyces unispora* resulted in significant quantitative changes (a higher total living algal cell concentration), but no qualitative alterations (unmodified kinetics of algal proliferation). The absence of significant differences between the rates of algal proliferation with and without the yeast, indicated by the very similar μ values, suggested that this yeast strain had no harmful effect on algal growth.

A strain-specific difference connected with a higher total algal cell concentration was observed in the spatial distribution of algae inside the

entrapping material. In agreement with the results of other authors (Zhang et al., 1998), both algal strains preferred the periphery of the beads when immobilized alone. However, the distribution pattern of *C. sorokiniana* 211.40c changed upon co-cultivation with *S. unispora* and became almost homogeneous, while the other algal strain, *C. saccharophyla* 3.80, co-immobilized with the yeast exhibited the pattern of algae immobilized alone.

The changed distribution offers a direct explanation for the higher total algal cell concentration, but the interaction between the two organisms is more difficult to sustain.

At present there is no direct evidence explaining why and how the applied yeast strain facilitates algal proliferation. Although other microorganisms, such as *Azospirillum brasilense*, which promote microalgal growth have been reported previously, using a similar cell entrapping experimental arrangement (Gonzalez and Bashan, 2000), this bacterium is well known as a plant growth factor producer, unlike *Saccharomyces unispora*.

Another possible determinative factor of cell growth (and indirectly of the location in the entrapping material) is the carbon source, CO_2 in the case of *Chlorella* strains. There are two possible CO_2 sources during the period of co-cultivation: (i) the external environmental CO_2 and (ii) the metabolic product of the partner microbe. Since the yeast could find the necessary conditions for growth, it must have produced CO_2 as a metabolic end-product. On the other hand, the appearance of yeast-specific products such as butyric acid indicates that the fermentation of glucose released by *Chlorella* took place, with CO_2 as an end-product of the process. This assumption tallies with the fact that, with *Chlorella sorokiniana* 211.40c, a relatively high glucose producer, both the total cell count and the level of yeast-specific products are higher than for the other algal strain.

To summarize, the results presented suggest that symbiotic green algae and their natural processes are suitable for the cultivation of heterotrophic organisms, and stable systems can be constructed with respect to the basic physiological characteristics of the partners.

This work highlights the potential for using symbiotic algae as a renewable source for the cultivation of microorganisms and is a preliminary step toward the generation of microbial ecosystems for biotechnological purposes.

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