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Enrichment and Isolation of Anoxygenic Phototrophic Bacteria in Winogradsky Column

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Özet: ***Anoksigenik fotosentetik bakterilerin wınogradsky kolonunda zenginleştirilmesi ve izolasyonu.*** Bu çalışmanın amacı Denizli Sarayköy'den alınan toprak ve çamur örneklerinden anoksigenik fototrofik bakteri izolasyonu ve saf kültür haline getirilen izolatın bakterioklorofil içeriğini belirlemektir. Bu amaçla örnekler, Winogradsky kolonunda, oda koşullarında 2-3 ay günışığında inkübe edilerek zenginleştirilmiştir. Phening's ortamında ondalık seyreltmeler yapılarak saf kültür elde edilmiştir. Elde edilen izolatın 400-1100 nm dalga boyu aralığında adsorbsiyon spektrumları taranmış ve 506-518, 806-810 ve 854-865 nm.'de maksimum absorbans verdiği bulunmuştur.

Anahtar Kelimeler: Anoksigenik fotosentez, Winogradsky kolonu, bakteriyoklorofil, Pfennig's ortamı, mikrobiyal süksesyon.

Abstract: The aim of the study is to isolate the anoxygenic photothophic bacteria from mud and soil obtained from Denizli Saraykoy and to determine bacteriochlorophyll content of the isolate. For this purpose samples were enriched under sunlight in Winogradsky column at room temperature for 2-3 months. Pure culture was obtained by decimal dilutions in Pfennig's medium and screened for maximum adsorption spectrum between 400-1100 nm. The absorption maxima of cell suspensions of isolated strains are 506-518, 806-810 and 854-865 nm.

Key Words: Anoxygenic photosynthesis, Winogradsky column, bacteriochlorophyll, Pfennig's medium, microbial succession.

Introduction

Aquatic ecosystems are very important reservoirs for carbon cycling. Photosynthesis is the crucial method for this aim. For many years, oxygenic photosynthesis of phytoplankton has been known to be the mechanism for forming primer productivity, but anoxygenic photosynthesis has not been well-discussed. (Nianzhi et al., 2003). Procaryotes that can convert light energy into chemical energy include the photosynthetic cyanobacteria, the purple and green bacteria and the "halobacteria" (archaea). The cyanobacteria conduct plant photosynthesis, called oxygenic photosynthesis; the purple and green bacteria conduct bacterial photosynthesis or anoxygenic photosynthesis; the extreme halophilic archaea use a type of nonphotosynthetic photophosphorylation mediated by bacteriorhodopsin to transform light energy into ATP (the only example in nature of non photosynthetic photophosphorylation) (Pfenning 1989). Anoxygenic photosynthetic bacteria are accounts for (11.3±1.7) % of total microbial community in euphotic zone. The bacteriochlorophyll a / chlorophyll a ratio is about 0.8 % in Northeastern Pacific. This percentage over global ocean is about 5%-10%. This means anoxygenic photosynthetic bacteria plays an important role in total primer productivity, so the global knowledge based on the fact that primary production is provided by phytoplankton is changed (Nianzhi et al. 2003).

Anoxygenic phototrophic bacteria are widely distributed in sediment and lake waters (Immhof, 1992). In addition to aquatic and marine ecosystems, photosynthetic primary productivity is also extremely high in extreme environments including acidic, alkaline thermal, cold and hipersaline habitats. Most of autothrophic activity in soda lakes, one of the extreme aquatic environments, is due to purple sulphur bacteria and Cyanobacteria (Jones et al. 1998).

There are several types of pigments distributed among various phototrophic organisms. Chlorophyll is the primary light-harvesting pigment in all photosynthetic organisms. Chlorophyll is a tetrapyrrole which contains magnesium at the center of the porphyrin ring. It contains a long hydrophobic side chain that associates with the photosynthetic membrane. Cyanobacteria have chlorophyll a, the same as plants and algae. The chlorophylls of the purple and green bacteria, called bacteriochlorophylls are chemically different than chlorophyll a in their substituent side chains. This is reflected in their light absorption spectra. Chlorophyll a absorbs light in two regions of the spectrum, one around 450nm and the other between 650 -750nm; bacterial chlorophylls absorb from 800-1000nm in the far red region of the spectrum (Brock and Madigan 1991).

Carotenoids are always associated with the photosynthetic apparatus. They function as secondary light-harvesting pigments, absorbing light in the blue-green spectral region between 400-550 nm. Carotenoids transfer energy to chlorophyll, at near 100 percent efficiency, from wave lengths of light that are missed by chlorophyll. In addition, carotenoids have an indispensable function to protect the photosynthetic apparatus from photooxidative damage. Carotenoids have long hydrocarbon side chains in a conjugated double bond

system. Carotenoids "quench" the powerful oxygen radical, singlet oxygen, which is invariably produced in reactions between chlorophyll and O_2 (molecular oxygen). Some nonphotosynthetic bacterial pathogens, i.e., *Staphylococcus aureus,* produce carotenoids that protect the cells from lethal oxidations by singlet oxygen in phagocytes (Brock and Madigan 1991; Imhoff and Trüper 1998).

The selection and development of sequential microbial populations in natural or disturbed systems is known as microbial succession. This succession occurs largely because the activities of initial populations bring about changes in the environment. These changes include decreases in available nutrients, alterations in pH or redox potential (E_h), disappearance of 0_2, and build-up of toxic metabolic products. The initial population becomes self-limiting due to the modifications of the environment which it mediated. At this point members of the population which were less dominant may find favorable growth conditions and emerge as the new dominant population. This population may further alter the environment leading to its own demise, and to the emergence of another different dominant population (Brock and Madigan 1991).

In natural ecosystems these changes may be rather subtle and go unnoticed to the untrained observer. However, in the laboratory a variety of model systems can be established which clearly demonstrate succession of microbial populations and changes in the environment brought about by certain microbial groups. The Winogradsky column provides a very convenient model system for laboratory demonstrations of microbial succession (Madigan 2003).

In this study, we report the composition and activities of phototropic bacteria in the mud and soil samples obtained from Denizli-Turkey isolation after enrichment in Winogradsky column and bacteriochlorophyll analysis. Cultures were grown Photothropically (anoxic/light) in 50 ml screw-capped bottles, containing Phenning's medium.

Material and Methods

To pack the column (40 x 5 cm) is to prepare the mud or soil samples as slurry having the consistency of heavy cream. The slurry can be enriched before packing the column. Calcium sulphate (1-2 % w/w) and calcium carbonate (1-2 % w/w) are added as sources of sulphur and CO_2, respectively. Finely shredded paper (a piece of newsprint about 100 cm^2) provides a long term carbon source and provides CO_2 and other organic substrates such as organic acids as it is mineralized (Tamer et al, 1989).

For isolation of photosynthetic bacteria Phenning' media in 50 ml bottles was prepared as following;

Solution 1*:* 0.83 g $CaCl_2.2H_2O$ in 2.5 liters H_2O. For marine organisms, add NaCl, % 5,2 g.

Solution 2: H_2O 67 ml; KH_2PO_4, 1 g; NH_4C1, 1 g; $MgCl_2.2H_2O$, 1 g; KC1, 1 g; 3 ml vitamin B_{12} solution (2 mg/100 ml H_2O); 30 ml trace element solution (1 liter H_2O, ethylene diamine tetraacetic acid, 500 mg; $FeSO_4.7H_2O$, 200 mg; $ZnSO_4.7H_2O$, 10 mg; $MnCl_2.4H_2O$, 3 mg; H_3BO_3, 30 mg; $CoCl_2.6H_2O$, 20 mg; $CuCl_2.2H_2O$, 1 mg; $NiO_3.6H_2O$, 2 mg; $Na_2MoO_4.2H_2O$, 3 mg, pH 3).

Solution 3: Na_2CO_3, 3 g; H_2O, 900 ml. Autoclave in a container suitable for gassing aseptically with CO_2.

Solution 4*:* $Na_2S.9H_2O$, 3 g; H_2O, 200 ml. Autoclave in flask containing a Teflon-covered magnetic stirring rod.

The bulk of solution 1 dispensed in 67-ml aliquots in 30 screw-capped bottles of 100 ml capacity and autoclaved with screw caps loosely on. The other solutions were also autoclaved in bulk. After autoclaving, all solutions were cool rapidly by placing the bottles in a cold water bath to prevent lengthy exposure to air. Solution 3 is then gassed with CO_2 gas until it is saturated (about 30 minutes; pH drops to 6.2) and added to cooled solution 2. 33 ml of this mixture was placed aseptically in each bottle containing solution 1. Solution 4 is partially neutralized by adding drop wise of 1.5 ml of sterile 2 M H_2SO_4 and stirring on a magnetic stirrer. 5 ml portions of solution 4 were added to each bottle, was filled the bottles completely with remaining solution 1, and tightly close. The final pH was adjusted to 6.7 - 7.2. It was stored overnight to consume residual oxygen before using (For some organisms, the sulfide concentration should be reduced. Use 2.5 ml instead of 5 ml of solution 4 per bottle). It may be necessary to "feed" the cultures with sulfide (solution 4) at weekly intervals for a period of two months, as the sulfide is used up during growth. To do this, we removed 2.5 or 5 ml of liquid from a bottle and refilled with an equal amount of sterile neutralized solution 4 (Brock and Madigan 1991).

Samples taken from Winogradsky column were done serial dilutions in Phening's medium and all bottles were incubated at adequate daylight and room temperature for 30 days. At time intervals (1 week), solution 4 was added to inoculated bottles to support anoxygenic photothophic bacteria.

The last serial diluted bottle showing bacterial growth was accepted as a pure culture and examined in a light microscope after gram staining.

After 30 days of incubation, 3.5 ml of culture from inoculated Phening's medium were homogenized with 5 g of sucrose and the absorption spectrum (400-1100 nm) was measured in a Shimadzu spectrophotometer using Pfennig's medium as the blank (Pansona et al. 2002).

Results and Discussion

In the Winogradsky column all the organisms are present initially in low numbers, but when the tubes are incubated for 2 to 3 months the different types of microorganism proliferate and occupy distinct zones where the environmental conditions favour their specific activities. The large amount of cellulose added to column and organic matter containing in mud initially promotes rapid microbial growth of heterotrophic bacteria which soon depletes the oxygen in the sediment and in the water column. Only the very top of the column remains aerated because oxygen diffuses very slowly through water.

Some cellulose-degrading *Clostridium* species start to grow when the oxygen is depleted in the sediment. All *Clostridium* species are strictly anaerobic because their vegetative cells are killed by exposure to oxygen, but they can survive as spores in aerobic conditions. They degrade the cellulose to glucose and then ferment the glucose to gain energy, producing a range of simple organic compounds (ethanol, acetic acid, succinic acid, etc.) as the fermentation end products (Blair and Anderson, 1999)

The sulphur-reducing bacteria such as *Desulfovibrio* can utilise these fermentation products by anaerobic respiration, using either sulphate or other partly oxidised forms of sulphur (e.g. thiosulphate) as the terminal electron acceptor, generating large amounts of H_2S by this process The H_2S will react with any iron in the sediment, producing black ferrous sulphide. This is why the bottom of column is frequently black. However, some of the H_2S diffuses upwards into the water column, where it is utilised by other organisms (Mudryk et al., 2000).

The diffusion of H_2S from the sediment into the water column enables anaerobic photosynthetic bacteria to grow. They are seen usually as two narrow, brightly coloured bands immediately above the sediment - a zone of green sulphur bacteria then a zone of purple sulphur bacteria (Brock et al., 1994).

The purple sulphur bacteria typically have large cells and they deposit sulphur granules inside the cells. The green sulphur bacteria have smaller cells and typically deposit sulphur externally.

The sulphur (or sulphate formed from it) produced by the photosynthetic bacteria returns to the sediment where it can be recycled by *Desulfovibrio* and chemolitotrophic *Thiobacillus* spp. part of the sulphur cycle in natural waters (Sigalevich et al. 2000)

Most of the water column above the photosynthetic bacteria is coloured bright red by a large population of purple non-sulphur bacteria. These include species of *Rhodopseudomonas*, *Rhodospirillum* and R*hodomicrobium*. These bacteria grow in anaerobic conditions, gaining their energy from light reactions but using organic acids as their carbon source for cellular synthesis. So they are termed photoheterotrophs. The organic acids that they use are the fermentation products of other anaerobic bacteria (e.g. *Clostridium* species), but the purple non-sulphur bacteria are intolerant of high H_2S concentrations, so they occur above the zone where the green and purple sulphur bacteria are found (Melis T., 2005).

The absorption maxima of cell suspensions of isolated strain which was gram positive coccus are 506-518, 806-810 and 854-865 nm, due to the blend of alternative spirilloxanthin series carotenoids, a typical carotenoid of the purple bacteria, and bacteriochlorophyl *a*, whose absorption maxima are at 375, 590, 800 - 810 and 830 - 890 nm (Imhoff and Trüper, 1989; Brock et al., 1994).

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