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Physico-chemical parameters influencing DNase activity of the cyanobacterium *Spirulina platensis*

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Abstract

The effects of temperature, Mg^{2+}, EDTA concentration and rinsing on extra- and intra-cellular DNase activity of *Spirulina platensis* strain SSP-14, were investigated. The results indicate that the tested strain contains very high extra- and intracellular DNase activity, which actually hinders the transfer of foreign gene(s) to *S. platensis*, a cyanobacterium with multiple economic potentials. The extracellular DNase activity could easily be removed by rinsing the cells with Zarrouk medium more than once. The intracellular DNase activity could also be inhibited by (1) removal of Mg^{2+}, (2) maintaining EDTA concentration above 1 mmol l^{-1}, and (3) manipulating below 0–4 °C, during all the incubation procedures. We suggest that, by using one or more of, or combining, all those experimental conditions, the chances of foreign DNA attempted to be introduced into *S. platensis* without being digested would be increased.

Key words: *Spirulina platensis* – DNase – extracellular nuclease

Introduction

In recent years, the potential uses of *Spirulina platensis*, a filamentous cyanobacterium, as a source of food and feed, have been pointed out (Becker 1986; Vonshak 1997). A reproducible and efficient genetic transformation system for this cyanobacterium, like those available in unicellular (Porter 1985) or other filamentous cyanobacteria (Wolk *et al.* 1984; Elhai 1993), has not been established yet. This situation may be a result of: (1) the absence of endogenous plasmids, (2) the high activity of extra- and intra-cellular DNases (deoxyribonucleases), in *Spirulina*. Although there may be some other reasons, the lacking of endogenous plasmids makes it impossible to construct a shuttle vector, an approach that was used for genetic transformation of unicellular cyanobacteria (Porter 1985) and also efficiently for some filamentous ones, e.g. *Anabaena* (Wolk *et al.* 1984). Such vectors are composed of endogenous plasmid fragments from both cyanobacteria and *E. coli,* hence they could prevent themselves from being degraded by restriction-modification systems and replicate autonomously within the two hosts. As a consequence, any foreign DNA, when used to transform *S. platensis,* could inevitably be digested before it is expressed independently or integrates into the genome. Thus lowering or removal of the DNase activity has, to a large extent, become the bottleneck of developing an efficient transformation system for *S. platensis*.

One approach to circumvent high DNase activity is to remove or shelter the DNase activity by genetic manipulation. Tragut *et al.* (1995) reported the identification of a gene encoding the methylase for Spa I, a restriction endonuclease with high activity in *S. platensis* strain pacifica. It is thus expected that newly-constructed vectors containing target gene(s) can be modified by this methylase, and thus preventing them from being cut when transformed into *S. platensis* cells. Yet this does not solve the problem of the three other endonucleases in this strain and in most cases, it is difficult to find the methylase corresponding to each endonuclease.

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We have tried to develop another approach of modifying physico-chemical parameters in order to generate a simple but reliable procedure to significantly reduce the DNase activity in the *S. platensis* cells.

Materials and methods

DNase preparation. An axenic culture of *Spirulina platensis* strain SSP-14 was prepared as described previously (Cao *et al.* 1999). One ml of exponentially grown cultures (OD_{560} 0.5–0.8) was centrifuged (12000 g, 15 min), and the cell-free supernatant was designated as the extracellular DNase solution (ED). After rinsing 5 times with cold (4 °C) Zarrouk medium (Zarrouk 1966), the pellet was resuspended in 1 ml of modified Zarrouk medium (supplemented with 40 mmol l^{-1} Tris-HCl, pH 8.0) and the cells were lysed by ultrasonication (50–60 w, 3 min) (Fisher Scientific, Model 550); the cell debris were removed by centrifugation as above at 4 °C, and the cell-free supernatant was denoted as the intracellular DNase solution (ID). DNA substrates employed in this work were pBR325 (Bolivar 1978) and λ DNA, which were both purified (Sambrook *et al.* 1989) and diluted to the concentration of 0.1 $\mu g\ \mu l^{-1}$.

Rinsing. After the ED solution was prepared, the pellet was resuspended and rinsed with 1 ml of Zarrouk medium by vigorous pipetting, for 3 times; the supernatant for each time was collected, respectively. The extracellular DNase activity assay, using pBR325 and λ DNA as the substrate, respectively, was conducted in Eppendorf tubes. The tubes were incubated at 28 °C for 1 and 24 h, respectively.

Temperature. The ID solution was prepared as above; the plasmid pBR325 was used as the substrate. The assay was carried out in Eppendorf tubes in which 3 μl DNA and 15 μl ID solution (or Zarrouk medium as the control) were mixed, and the tubes were incubated at 0, 4, 16, 28 and 37 °C, respectively, for 1 h.

Mg^{2+}. In order to study the effect of Mg^{2+} concentration on DNases, an exponential culture was harvested and resuspended in Mg^{2+}-free Zarrouk medium and cultivated to the exponential stage, as described previously (Cao *et al.* 1999); then Mg^{2+}-free ED and ID solutions were prepared as above and the DNase activity of the preparation was evaluated. The reaction was performed at 28 °C for 1 h .

EDTA. As in the Mg^{2+} test, the pellet was resuspended in EDTA-free medium and the culture was cultivated to the exponential stage; then EDTA-free ID and ED solutions were prepared. The digestion assay was conducted in different concentrations of EDTA at 28 °C for 1 and 24 h, respectively.

Peak areas. The peak area of every DNA band on the gel was measured by means of NIH Image Analysis software.

Results

Rinsing

The effect of rinsing the cells on the extracellular DNase activity is demonstrated in Fig. 1. Lanes 4–6, representing 1st, 2nd and 3rd rinsing extracts, respectively, had almost the same peak areas (95%, 96% and 98%, respectively) as lane 1 (control, with a peak area of 100%), even when the digestion time was extended to 24 h (Fig. 1 A, B), while both the culture (lane 2) and ED solution (lane 3) partially (1 h treatment) or even completely (24 h treatment) degraded the plasmid pBR325,

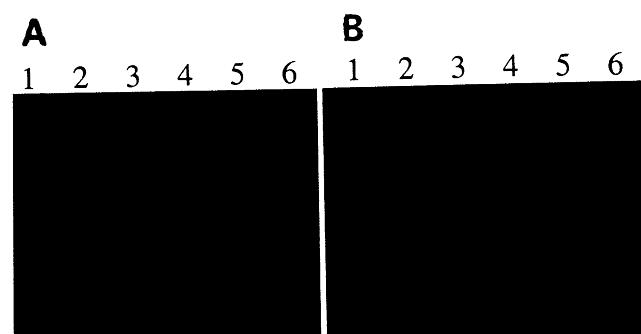

Fig. 1. Effects of rinsing on the extracellular DNase activity (pBR325 as the substrate). (A) treated for 1 h and (B) treated for 24 h. Lane 1 (control): 15 μl Zarrouk medium + 3 μl DNA; lane 2: 15 μl culture + 3 μl DNA; lane 3: 15 μl ED solution + 3 μl DNA; lane 4: 15 μl 1st rinsing ED solution + 3 μl DNA; lane 5: 15 μl 2nd rinsing ED solution + 3 μl DNA; lane 6: 15 μl 3rd rinsing ED solution + 3 μl DNA.

Fig. 2. Effects of rinsing on the extracellular DNase activity (λ DNA as the substrate). Lane 1 (control): 15 μl Zarrouk medium + 3 μl DNA; lane 2: 15 μl culture + 3 μl DNA; lane 3: 15 μl ED solution + 3 μl DNA; lane 4: 15 μl 1st rinsing ED solution + 3 μl DNA; lane 5: 15 μl 2nd rinsing ED solution + 3 μl DNA.

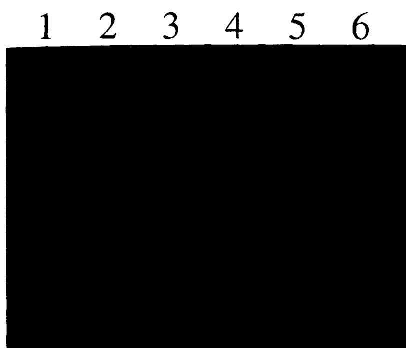

Fig. 3. Effects of temperature on the intracellular DNase activity (pBR325 as the substrate). Lane 1 (control): 15 µl Zarrouk medium + 3 µl DNA; lanes 2–6: 15 µl ID solution + 3 µl DNA, which have been incubated at 0, 4, 16, 28 and 37 °C, respectively, for 1 h.

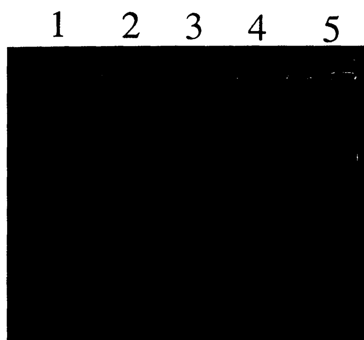

Fig. 4. Effects of Mg^{2+} on DNase activity (λ DNA as the substrate). Lane 1 (control): 15 µl Mg^{2+}-free Zarrouk medium + 2 µl DNA; lane 2: 15 µl ID solution + 2 µl DNA; lane 3: 15 µl ID solution + Mg^{2+} +2 µl DNA; lane 4: 15 µl ED solution + 2 µl DNA; lane 5: 15 µl ED solution + Mg^{2+} + 2 µl DNA. In lanes 3 and 5, Mg^{2+} was added to a final concentration of 0.8 mmol l^{-1}, as much as in Zarrouk medium.

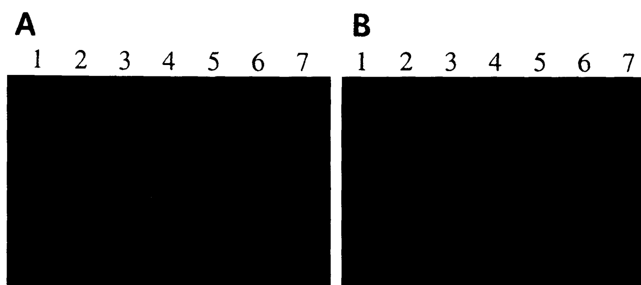

Fig. 5. Effects of EDTA concentrations on the intracellular DNase activity (pBR325 as the substrate). (A) treated for 1 h and (B) treated for 24 h. Lane 1 (control): 7.5 µl EDTA-free Zarrouk medium + 1 µl sterile ddH_2O + 1.5 µl DNA; lane 2: 7.5 µl ID solution + 1 µl ddH_2O + 1.5 µl DNA; lane 3: 7.5 µl ID solution + 1 µl 2.5 mmol l^{-1} EDTA + 1.5 µl DNA; lane 4: 7.5 µl ID solution + 1 µl 5 mmol l^{-1} EDTA + 1.5 µl DNA; lane 5: 7.5 µl ID solution + 1 µl 7.5 mmol l^{-1} EDTA + 1.5 µl DNA; lane 6: 7.5 µl ID solution + 1 µl 10 mmol l^{-1} EDTA + 1.5 µl DNA; lane 7: 7.5 µl ID solution + 1 µl 100 mmol l^{-1} EDTA + 1.5 µl DNA.

implying that the extracellular DNase was easily removed by rinsing the cells with Zarrouk medium more than once. When λ DNA was used as the substrate and treated for 1 h (Fig. 2), similar results were obtained.

Temperature

The effect of low temperature on DNase activity is demonstrated in Fig. 3. Temperature had remarkable effects on DNase activity. Lanes 2 and 3, representing temperatures 0 °C and 4 °C, respectively, had almost the same peak areas (99% and 98%) as the control (100%, lane 1); however, no bands were detected in lanes 4–6, when the incubation temperature was raised to 16, 28 or 37 °C as indicated. Consequently incubation at low temperature (0–4 °C) resulted in a complete inhibition of the DNase activity, while incubation at temperature above 16 °C resulted in complete degradation of the DNA.

Mg^{2+}

The effect of Mg^{2+} on DNase activity is shown in Fig. 4. In lanes 1–5, the peak area of each λ DNA band is 100% (control), 98%, 6%, 99% or 68%. In lanes 2 and 4, digestion was inhibited due to the absence of Mg^{2+}; in lanes 3 and 5, both intracellular and extracellular DNase activities were recovered because of the addition of Mg^{2+}. But the increase of ID was much greater than that of ED. The same result was achieved when pBR325 was used as the substrate (data not shown).

EDTA

The effect of EDTA on DNase activity is demonstrated in Figs. 5 and 6. When digested for 1 h, the peak areas in lanes 6 and 7 were almost the same as in lane 1 (control), that in lane 5 only 20%, but in lanes 2–4, no bands detectable (Fig. 5A). When digestion time was extended to 24 h (Fig. 5B), no pBR325 bands were detected in lanes 2–5, and a very weak band in lane 6 and almost no change in peak area in lane 7. The results showed that DNase activity was inhibited with the increasing the concentration of EDTA, and the inhibition was sufficiently effective when EDTA concentration was 1 mmol l^{-1} (final concentration) (1 h) or 10 mmol l^{-1} (24 h).

Discussion

The effects of four physical and chemical parameters: rinsing, temperature, Mg^{2+} and EDTA concentration, on both extra- and intra-cellular DNase activities of *S. platensis* strain SSP-14, were examined. The results pre-

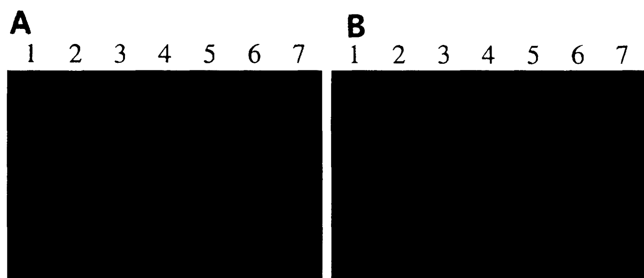

Fig. 6 Effects of EDTA concentrations on the extracellular DNase activity (pBR325 as the substrate). (A) treated for 1 h and (B) treated for 24 h. Lane 1 (control): 7.5 µl EDTA-free Zarrouk medium + 1 µl sterile ddH_2O + 1.5 µl DNA; lane 2: 7.5 µl ED solution + 1 µl ddH_2O + 1.5 µl DNA; lane 3: 7.5 µl ED solution + 1 µl 2.5 mmol l^{-1} EDTA + 1.5 µl DNA; lane 4: 7.5 µl ED solution + 1 µl 5 mmol l^{-1} EDTA + 1.5 µl DNA; lane 5: 7.5 µl ED solution + 1 µl 7.5 mmol l^{-1} EDTA + 1.5 µl DNA; lane 6: 7.5 µl ED solution + 1 µl 10 mmol l^{-1} EDTA + 1.5 µl DNA; lane 7: 7.5 µl ED solution + 1 µl 100 mmol l^{-1} EDTA + 1.5 µl DNA.

sented indicated that, although the tested strain contained very high extra- and intra-cellular DNase activities, by modifying each of the parameters, the DNase activity could partially or even completely be inhibited. It is suggested that by applying these conditions, a genetically-engineered DNA construct (vector), when introduced into *S. platensis,* could be protected and remain undigested before the possible integration into the genome. Tragut *et al.* (1995) had made an effort to remove or shelter the DNase activity by genetic manipulation, using a gene encoding the methylase for Spa I, a restriction endonuclease present in *S. platensis* strain pacifica. However, in most cases, it is difficult to identify the methylase corresponding to each endonuclease. It is much easier and more efficient to use physical and chemical methods such as what were reported in this paper. Although this kind of removal is temporary, yet it may be enough to allow the foreign gene(s) to integrate into the recipient's genome.

It is believed that the presence of restriction endonucleases may be the most formidable obstacle in the genetic transformation of cyanobacteria (Vachhani and Vonshak 1997), much work was done and various kinds of endonucleases have been identified and reported in many cyanobacteria (De Marsac and Houmard 1987; Takahashi *et al.* 1996). As reported by Kawamura *et al.* (1986), three restriction endonucleases, Spl I, Spl II and Spl III are present in *S. platensis* subspecies *siamese,* of which Spl I is a new endonuclease and the other two are the isoschizomers of enzymes reported in other organisms. Recently, Tragut *et al.* (1995) characterized four endonucleases in *S. platensis* strain pacifica, which are all isoschizomers reported previously. In the strain SSP-14 studied in this work, we found evidences for the existence of both exonuclease and endonuclease, and the exonuclease activity is very high. Unlike other reports in which several sharp bands were shown after digestion by cell-free extracts (Tragut *et al.* 1995; Takahashi *et al.* 1996), we did not detect smaller bands on agarose gels when the substrate DNA was digested. On the contrary, we only observed the decrease in the original band. This is a typical digestion pattern for exonucleases. In order to linerize a supercoiled plasmid such as pBR325, an endonuclease is required, only then exonucleases could digest it into very short segments, too short to be detectable on gels. However, whether there is only one DNase in *S. platensis* which possesses both endonuclease and exonuclease activities, as reported in Colorado potato beetle (Borodin and Konichev 1997), needs further investigation.

It was reported that some cyanobacteria produce extracellular or cell wall-associated DNase (Wolk and Kraus 1982; Soper and Reddy 1994), denoting that extra- and intra-cellular DNases have obligate distribution and have different composition and function. In the strain SSP-14, it seems that there was no difference between extra- and intra-cellular DNases. As shown in Figs. 5 and 6, some differences in the digestion patterns for 1 h treatments (Figs. 5A and 6A) could be found. When the digestion time was extended to 24 h, no differences were observed (Figs. 5B and 6B). The differences may result from the fact that extra- and intra-cellular DNases had different concentrations. The same results were also observed in the temperature treatment (data not shown). To examine whether the extracellular DNase is secreted into the medium or associated with the cell wall, the DNase activity in the culture supernatant was tested (Takahashi *et al.* 1996). A cell wall-associated DNase was released from the cells of *Cyanothece* by rinsing, it was undetectable in the culture supernatant, indicating that it is not secreted into the medium (Soper and Reddy 1994). As shown in Fig. 1B lane 3, the extracellular DNase from the culture supernatant could completely digest the substrate after 24 h treatment; when it was rinsed, even only one time, its activity was completely removed, showing that the extracellular DNase is secreted from the cells into the medium and is not cell wall-associated. When Mg^{2+} was removed from the medium and from the digestion mixture, the activity of both extra- and intra-cellular DNases was inhibited, and when it was restored, the activity also recovered (Fig. 4), indicating that the DNase activities are Mg^{2+}-dependent. This was also verified in the EDTA test, in which the addition of EDTA to the reaction mixture to a concentration above 1 mmol l^{-1} efficiently inhibited the DNase activity, indicating that a bivalent cation was essentially required. This is Mg^{2+}.

Acknowledgements

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