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original scientific paper

Direct Detection of Extracellular Deoxyribonucleases in Different Strains of *Streptomyces rimosus*

Dušica Vujaklija, Jelena Žafran, Andreja Mikoč and Vera Gamulin*

Department of Molecular Genetics, »Ruđer Bošković« Institute, Bijenička c. 54, 10000 Zagreb, Croatia

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Summary

***Streptomyces rimosus**, an oxytetracycline producer, excretes from the cell small deoxyribonuclease (DNase) with M_r=19 000. The presence of the extracellular enzyme was confirmed by three rapid methods specially developed for this purpose. These tests can be employed for **in vivo** and **in vitro** detection of DNases. When bacteria were cultivated on solid medium with addition of DNA and toluidine blue, red zone appears around the colonies with extracellular DNase activity. Using this approach we found among **S. rimosus** strains, R6-779 with the highest activity and no activity in the strain R6-554; therefore this strain is suitable for complementational cloning of the gene. Activity of the DNase in the liquid media was detected in the microtest which contained only few μL of the filtrate mixed with 5 μg of DNA. Degradation of the DNA was monitored by agarose gel electrophoresis. Electrophoresis of unconcentrated filtrates on denaturing polyacrylamide gels containing high molecular weight DNA, allowed direct detection of the 19 000 Da enzyme. Activity of the enzyme changes in different fermentation media, but it is not influenced by addition of DNA. Highest activity was observed in the fermentation medium GR2d.*

Keywords: Streptomyces rimosus, endonuncleases, extracellular deoxyribonucleases

Introduction

Streptomycetes are Gram-positive mycelial soil bacteria with complex life cycle, which produce hundreds of biologically active compounds *(1–3)* and are therefore considered as the most important group of industrial microorganisms. Bacteria from the genus *Streptomyces* release in the extracellular milieu a variety of hydrolytic enzymes *(4)*, among them several DNases and RNases, which were detected in the culture media of different *Streptomyces* spp. long ago *(5,6)*. Most DNases produced by *Streptomyces* spp. are type II restriction endonucleases. These enzymes have been extensively used in molecular biology and biochemistry research laboratories. However, detailed biochemical analyses of unspecific DNases were never performed. One exception is the DNase from *S. rimosus* K-OK-02 (used for the production of oxytetracycline – OTC in KRKA, Chemical and Pharmaceutical Works, Novo Mesto, Slovenia). This enzyme was purified to homogeneity and several biochemical parameters were analysed. The enzyme is unspecific endodeoxynuclease with M_r of about 21 000 *(7)*.

Unspecific DNases, like the one present in *S. rimosus* K-OK-02 were never commercially prepared from *Streptomyces* spp. although these bacteria are a very convenient source for the quick and cheap isolation of DNases. In the culture collection of *Streptomyces* spp. in PLIVA, Chemical and Pharmaceutical Company, Zagreb, different *S. rimosus* strains exist. We decided to screen available strains with the aim to define the most convenient DNase producer and to find the growth conditions which are optimal for the release of the DNase activity in the culture media. For that reason we developed quick and cheap methods for the detection and analysis of extracellular DNases activity.

Experimental

Bacterial strains

Oxytetracycline-producer *Streptomyces rimosus* R6, its mutants: R6-593 (*thy*, otr^R, RP3), -554 (*rib*12, otr^R), -554w (*rib*12, *otc*, otr^R, RP3), -779 (*otc*, otr^R, RP3) and *S. rimosus*

* Corresponding author

ATCC 10970 were obtained from J. Pigac (PLIVA, Chemical and Pharmaceutical Company, Croatia). *S. lividans* 1326 (SLP2, SLP3) and TK64 (*pro*-2, *str*-6) were obtained from D. A. Hopwood, John Innes Institute, UK.

Media and growth conditions

Streptomycetes were grown in CRM medium *(8)* or TCA medium containing in g/L: 2 Bacto peptone; 0.5 $MgCl_2 \cdot 6H_2O$; 2 yeast extract; 10 glucose; 2.5 casamino acids. pH was adjusted to 7.2. M4 and GR2d media were obtained from PLIVA as undefined industrial media. For solid media, 20 g/L of Difco-agar was added to liquid CRM or TCA media.

Preparation of the filtrates and cell lysate

For detection of DNase activity in the liquid media, the filtrates were collected at different stages of bacterial growth, after removing the mycelia by centrifugation at $5000 \times g$, 10 min.

For the preparation of the cell lysate mycelia were harvested as described above, washed with 10 mM Tris·HCl, pH=7.5, 1 mM EDTA, 1 mM DTT and 10% glycerol and sonicated in the same buffer supplemented with phenylmethylsulfonyl fluoride (0.5 mM). Cell debris was removed by centrifugation at $12000 \times g$ for 25 min and the supernatant was tested for the presence of DNase activity.

Detection of the DNase activity

a) Agar test for DNase

The method for detection of DNases, previously described in GibcoBRL (Cat. No. 152-01580), was modified for testing the productivity of extracellular DNase in *Streptomyces* strains. High molecular weight DNA prepared from fish sperm (Serva) was incorporated in the solid test media (400 μg/mL) supplemented with toluidine blue (0.01%). DNase activity in the plates can be detected as the appearance of the red zone surrounding the positive colonies in an otherwise blue-coloured medium.

b) Agarose gel electrophoresis test

1–5 μL of filtrate was mixed with 5 mg of high molecular weight DNA in the final volume of 20 μL of 50 mM Tris.HCl, pH=7.5 and 5 mM $MgCl_2$. Reaction was carried out at 37 °C and after 20–90 min the reaction was stopped by addition of 20 μM EDTA. The samples were applied onto 0.7% agarose gel and degradation of DNA was visualised by the standard gel electrophoresis method (9).

c) SDS-PAGE electrophoresis test

The proteins from the filtrates or cell lysates were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described *(10)*. Separating gel containing 10 μg/mL of high molecular weight DNA was prepared as described *(11,12)*. After the electrophoresis, gel was incubated at room temperature in the renaturation buffer (40 mM Tris · HCl, pH=7.5, 15 mM $MgCl_2$) for 30 min (5 times) to remove SDS and then left overnight in the same buffer supplemented with 4 mM $CaCl_2$. The activity of the DNases was visualised directly after staining the gel with EtBr (the transparent band on the red-orange fluorescent background).

Results

We applied three different methods for the quick and direct detection of the extracellular DNase activity in *S. rimosus* strains: agar-test, agarose gel electrophoresis test and SDS-PAGE electrophoresis test.

Agar test for DNase

The simplest and the fastest way to detect visually the deoxyribonuclease activity of *S. rimosus* is to grow the colonies on the indicator agar as described in Experimental. Accumulation and release of the DNases from the cells in the extracellular milieu can be easily monitored by the change of the colour of the solid media.

The results of one representative agar test are shown in Fig. 1. Using this approach we were able to detect the production of extracellular DNases only in some of the examined *S. rimosus* R6 strains. The strain which showed the highest DNase activity by this method is *S. rimosus* R6-779, mutated in the pathway of OTC production. Similar results have been obtained with the parental strain *S. rimosus* R6. We were not able to detect any activity in the strains R6-554 and R6-554w. In parallel we checked the production of DNases by *S. lividans* TK64 and 1326 strains. With this method only *S. lividans* 1326 showed moderate activity of the extracellular DNases (data not shown). Three different solid media, TCA, CRM and LB, were used for these detections. The results proved that TCA medium is the most suitable for the agar test detection of the DNase activity.

The same test was applied for the analysis of DNase activities present in the filtrates of the exponentially

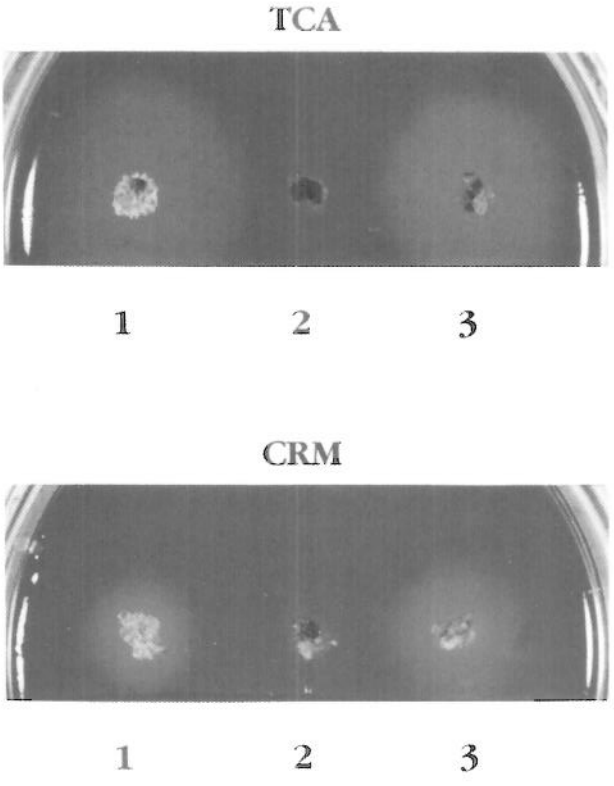

Fig 1. Agar-test for detection of extracellular DNase in *Streptomyces* strains. *S. rimosus* R6-779 (1), -554 (2), and R6 (3) were analysed on two different media, TCA and CRM.

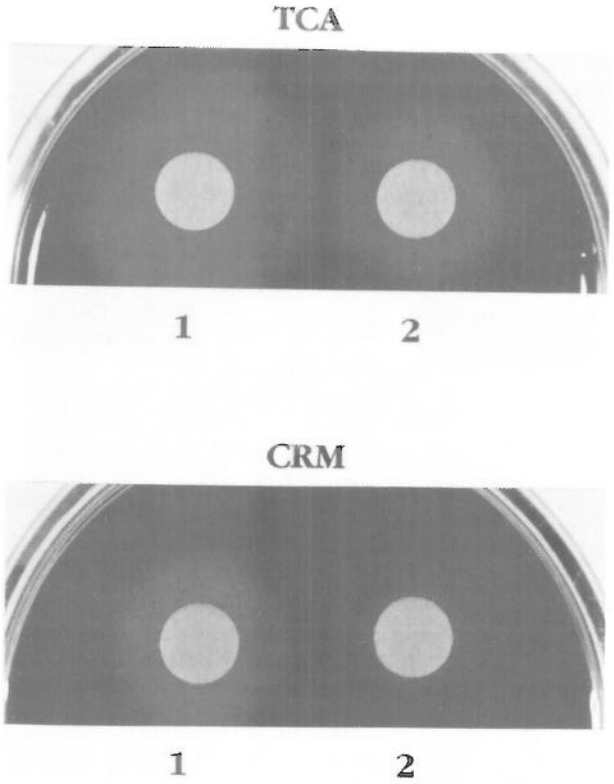

Fig. 2. Agar-test for detection of extracellular DNase present in the filtrate. *S. rimosus* R6-779, was grown in CRM medium. 50 μL of 5× concentrated (1) and 50 μL of nonconcentrated filtrates (2) were applied on the paper discs and the plates were incubated overnight at 28 °C.

growing cells in the liquid media. Aliquots of the filtrates were dropped on the sterile paper discs and incubated on the indicator agar plates. Red haloes appeared around paper discs after overnight incubation. The results are shown in Fig. 2.

Agarose gel electrophoresis test for DNase

In order to confirm our preliminary results obtained on indicator agar plates we developed microtest for the monitoring of DNase activity based on agarose gel electrophoresis method. This allowed direct visualisation of the degradation of high molecular weight DNA as a consequence of DNase activity. The bacteria were grown in TCA or CRM liquid medium until the cells reached stationary phase. Only few microliters of the cell filtrates mixed with the high molecular weight DNA were sufficient to detect the presence of DNases. This test can be done in parallel with numerous samples.

Preliminary results of the agar test were confirmed with this method as shown in Fig. 3. The strain R6-779 showed, as expected, the highest activity of the extracellular DNases. Therefore, this strain was used for further experiments. Using the same approach we checked the time and media dependent DNase activity. Bacteria were grown in CRM or TCA liquid medium for 6, 12, 18, 24, 32 and 40 hours. The DNase activity was checked in the presence or absence of 10% sucrose in the media. The results are shown in Fig. 4. The DNase activity is accumulated in the medium and the appearance of the en-

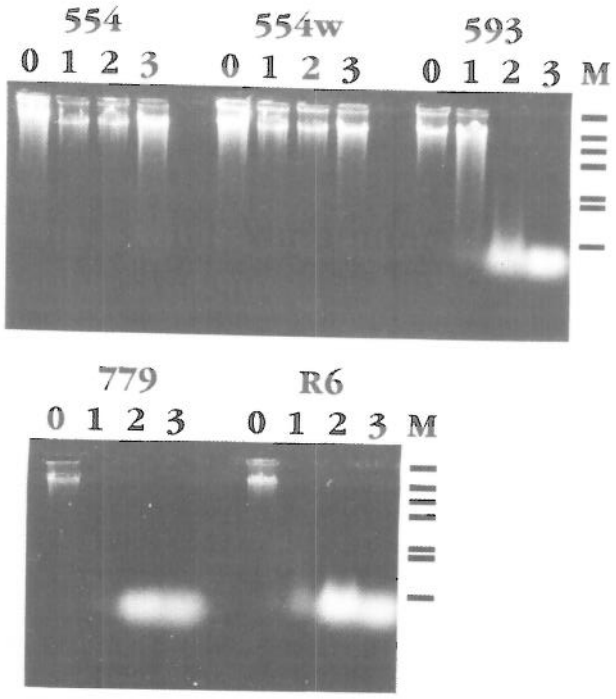

Fig. 3. Agarose-gel electrophoresis test. Filtrates were obtained from the *S. rimosus* R6 strains grown in the CRM medium for 48 hours. 2 μL of filtrates mixed with high molecular DNA, were incubated for 0 (0), 15 (1), 30 (2) and 60 (3) minutes. λ DNA digested with *Hind*III was used as control (M).

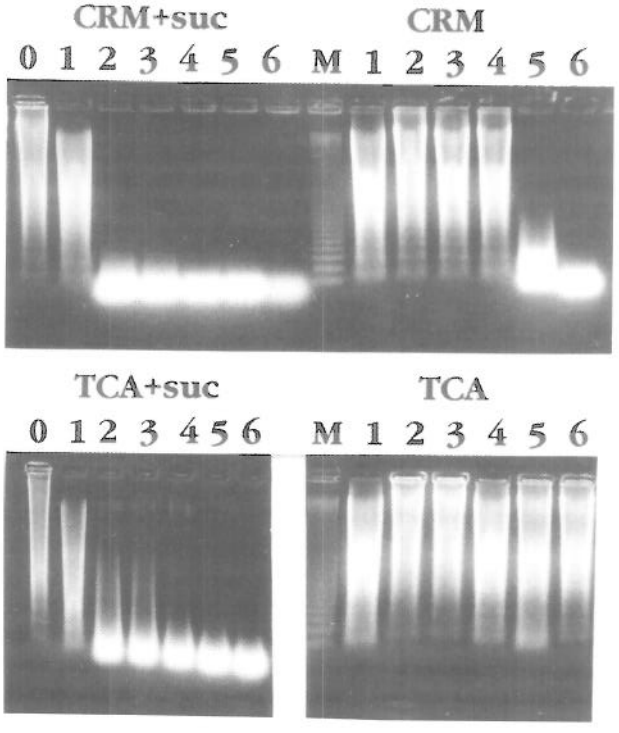

Fig. 4. Agarose-gel electrophoresis test for the analysis of DNase activity present in the filtrates obtained from *S. rimosus* R6-779, grown in TCA or CRM with or without sucrose. The filtrates were collected after 6 (1), 12 (2), 18 (3), 24 (4) 32 (5) and 40 (6) hours. The amount of the filtrate used for reaction varied from 1.5 to 10 μL, depending on the amount of cell biomass. The reaction time was 90 minutes. *Tenebrio molitor* DNA digested with *Eco*RI was used as control (M).

zyme can be observed after 12 hours of the cell growth. The addition of sucrose allowed us to detect DNase activity, in the filtrates, at the earlier stage of bacterial growth in both media. However, the DNase activity in CRM medium was significantly higher than in TCA medium.

We also checked if the addition of the DNA in the medium (0.1 mg/mL) had any influence on the DNase activity and found no difference (data not shown). Comparison of the DNase activity in the cell lysates and filtrates is shown in Fig. 5. The activity in the filtrates was considerably higher indicating that the enzyme specifically accumulates extracellularly.

S. rimosus R6-779 has grown very well in two industrial undefined media, M4 and GR2d, where the DNase activity was very high (Fig. 5).

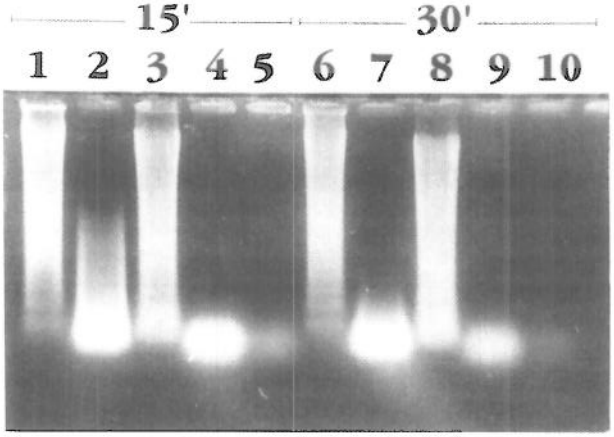

Fig. 5. Agarose-gel electrophoresis test for detection of DNases from the filtrates and lysates prepared from *S. rimosus*. Strain R6-779 was grown in CRM (2,3,7,8), M4 (4,9) and GR2d (5,10) media. 4 μL of filtrate (2,4,5,7,9) and the same volume of 2× concentrated cell lysate (3,8) were incubated with high molecular DNA for 15 or 30 minutes.

Using ribosomal RNA as a substrate we were unable to detect any RNase activity in the filtrates (data not shown).

SDS-PAGE test for DNase

The filtrates of *S. rimosus* R6-779 and -593 strains were analysed for the presence of DNase activity with SDS-PAGE test. The results are shown in Fig. 6A. Only one band in both strains was detected at the position of approximately 19 kDa. In agreement with the previous results the filtrate from strain R6-779 showed higher activity. As a control we used 4 μg of commercial DNase from bovine pancreas (Serva) of the known size of 31 kDa.

The same method was applied to compare the presence of DNase in the filtrate and lysate of *S. rimosus* R6-779 (Fig. 6B). The activity of DNase in the filtrate was higher in comparison with the DNase activity present in the lysate. In the cell lysate we were able to detect one intracellular DNase of the size estimated to be around 25 kDa.

In order to detect proteins present in the filtrates, SDS-PAGE gel was stained with silver salts. Several defined protein bands were visible after staining, but not at the position of the DNase (around 19 kDa) indicating that the mass concentration of the DNase in the filtrate is less than 1 ng/μL (data not shown).

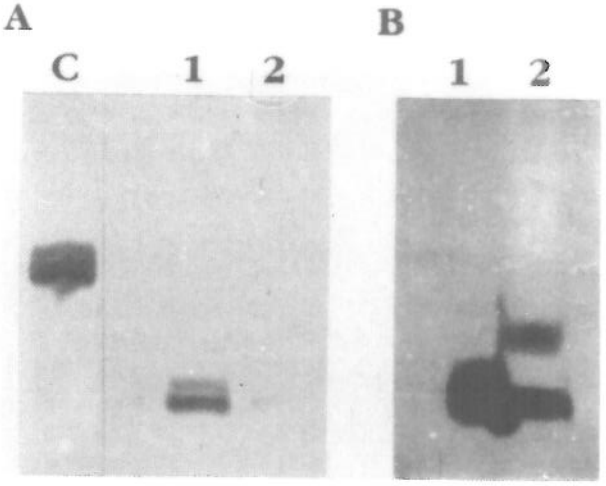

Fig. 6. SDS-PAGE test of DNase activity. A) 8 μL of the filtrates from strain R6-779 (1) and -593 (2) obtained from the stationary phase of bacterial culture, grown in CRM media, were analysed. 4 μg of commercial DNase from bovine pancreas (C) was used as control. B) 15 μL of filtrate (1) and cell lysate (2) obtained from R6-779 as described above were used for the analysis.

Discussion

Extracellular DNases with M_r of about 20 000 are identified in many different *Streptomyces* species *(6)*. This and the fact that we faced significant problems performing plasmid isolation and protoplast transformation prompted us to examine DNase production by parental strain, *S. rimosus* R6 and its mutants. For that purpose we developed three different methods for direct and easy detection of DNase activity. With these tests we detected extracellular DNase produced by almost all tested strains. Our estimation of its relative molecular mass is 19 000 and is based on the direct visualisation on the SDS-PAGE gel. Biochemicaly the best characterised DNase from *S. rimosus* K-OK-02 has M_r of about 21 000. Amino acid sequence of its N-terminus *(7)* did not reveal any homology with known protein sequences from related microorganisms. Probably unspecific DNases with similar molecular weight, detected up to now in many different *Streptomyces* spp., represent proteins with highly conserved primary structure. Therefore, the gene(s) encoding DNases of about 20 kDa in different *Streptomyces* spp., could be interesting for the analysis and cloning, and our tests can be very useful for that purpose.

Change of the phenotype, i.e. appearance of the red halo on the indicator plate after transformation of non-producing strain, is a very elegant way to identify desired transformants. We identified strain R6-554 as a good candidate (host strain) for the cloning of DNase gene (Fig. 1).

Of all available *S. rimosus* R6 strains, strain R6-779 always showed the highest activity of extracellular DNase (Fig. 3). However, this activity varied consider-

ably in different media indicating that the enzyme is inducible. We were not able to identify the inducer(s). The highest activity of the DNase was observed in commercial media of unknown composition, named M4 and GR2d (Fig. 5). Addition of the substrate for DNases, high molecular weight DNA, in the liquid medium did not influence the amount/activity of the enzyme. Presence of the sucrose in culture media significantly improved DNase activity. This effect could be attributed to the enhanced transport of the enzyme into extracellular milieu. Our results speak strongly in favour of DNase detected in the culture media of *S. rimosus* R6 as a real extracellular enzyme and not a »leaky« enzyme. DNase activity in cell filtrates was always considerably higher than the activity in the corresponding cell free extracts. Furthermore, only one DNase with M_r of around 19 000 is excreted outside the cell (see Fig. 6A). SDS-PAGE electrophoresis analysis identified only one additional DNase with M_r of about 25 000 in the cell free extracts (Fig. 6B). It is however very possible that additional DNase(s) exists in the cell and was not detected by this quick and direct method. Low intracellular concentration of the enzyme, or different renaturation kinetics of different proteins, can easily be reasons for the failure in their detection.

For similar reason it is very difficult to discuss the amount of the enzyme present in different filtrates, because we only detected DNase activity and never a DNase as a protein. Enzyme activity was measured directly in unpurified filtrates and the assay conditions varied accordingly. Therefore, one can not directly correlate the activity with the amount of the enzyme. The possibility that in filtrates from different culture media the same amount of the enzyme shows different activity is very possible. The precise measurement of the DNase activity in filtrates was not possible. Filtrates contain hundreds of (mostly unknown) compounds and monitoring of the DNA degradation by the increase of the UV absorbance at 260 nm is very inaccurate. Nevertheless, we can say that the enzyme is present at the rather low concentration in the filtrates even when its activity was found to be very high. Proteins from 5–25 μL of different filtrates were separated on SDS-PAGE gel and stained with silver. Several defined protein bands are visible after staining, but not at the position of the DNase (around 19 000) indicating that the mass concentration of DNase is less than 1 ng/μL.

RNase activity was never detected in the filtrates of *S. rimosus* R6 strains which is very important for the potential commercial use of this extracellular DNase. Many commercial DNases are not RNase free and therefore not suitable for the application in a variety of recombinant DNA methods.

Quick detection of the DNases activity using indicator agar plates and paper disks, or microtest on agarose gel, can be generally applied to diagnose the presence of DNases in small samples from any source.

Acknowledgment

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Izravna detekcija ekstracelularnih deoksiribonukleaza u različitim sojevima bakterije *Streptomyces rimosus*

Sažetak

Bakterija ***Streptomyces rimosus****, proizvođač oksitetraciklina, izlučuje u ekstracelularni medij deoksiribonukleazu (DNazu) relativne molekularne mase 19 000. Autori su razradili tri brze metode za detekciju ove DNaze* ***in vivo*** *i* ***in vitro****, te ispitali nekoliko sojeva* ***S. rimosus****. Najbrži je način detekcije ekstracelularnih DNaza uzgoj bakterija na krutoj podlozi u koju je dodana DNA, te toluidin plavilo. Oko kolonija koje izlučuju DNazu u podlogu i razgrađuju DNA pojavljuje se krug crvenog obojenja. Tom su metodom autori utvrdili da soj R6-779 bakterije* ***S. rimosus*** *izlučuje najviše DNaze dok soj R6-554 ne pokazuje nikakvu aktivnost, pa je stoga pogodan za kloniranje gena komplementacijom. Aktivnost DNaze u tekućoj podlozi detektirana je praćenjem smanjenja*

prosječne veličine DNA supstrata elektroforezom na gelu agaroze. Za utvrđivanje prisutnosti DNaze ovim mikrotestom dovoljno je za reakciju uzeti nekoliko μL staničnog filtrata i do 5 μg visokopolimerne DNA. Izravna detekcija DNaze, relativne molekularne mase 19 000, provedena je elektroforezom neugušćenih filtrata na denaturirajućim poliakrilamidnim gelovima u koje je dodana visokopolimerna DNA. Aktivnost enzima varira u različitim fermentacijskim medijima, ne mijenja se dodatkom DNA u medij, a najveća je u industrijskoj podlozi GR2d.