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ORIGINAL PAPER

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Biosynthesis of a thermostable gellan lyase by newly isolated and characterized strain of *Geobacillus stearothermophilus* 98

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Abstract The thermophilic strain able to degrade gellan was isolated from Bulgarian hot spring. According to its morphological and biochemical properties and by partial sequencing of its 16S rDNA, it was classified as *Geobacillus stearothermophilus*. It grew in a synthetic medium with gellan as the only carbon source with a specific growth rate of 0.69 h^{-1} and generation time of 60 min. The strain produced thermostable gellan lyase extracellularly during exponential phase. Its synthesis was inducible; the enzyme was not registered in culture liquid without gellan. The enzyme activity was increased tenfold in conditions of continuous cultivation compared to data from batch fermentations and enzyme productivity was almost sixfold higher. The enzyme showed optimal activity at 75°C in a very large pH area 4–8.5. This enzyme is the first reported thermostable gellan lyase, its residual activity was 100% after 24 h incubation at 60°C and its half-life was 60 min at 70°C.

Keywords *Geobacillus stearothermophilus* · Thermostabile gellan lyase · Continuous cultivation

Introduction

Microbial polysaccharides are a relatively new group of polysaccharides. They suggest novel properties for exploitation by biotechnological industry and also fast and directed polysaccharide synthesis. Gellan (linear tetrasaccharide repeating sequence consisting of D-glucose–D-glucuronic acid–D-glucose–L-rhamnose unit) is a relatively recently described microbial heteropolysaccharide, belonging to the group of sphingans (producing bacteria were shown to be members of the genus *Sphingomonas* (Pollock 1993). It possesses interesting properties such as high viscosity and high thermo-, acid- and enzyme resistance that could offer it for interesting commercial application as a gelling agent, thickener or stabilizer in food and pharmaceutical industries, and cosmetics (Hashimoto et al. 1999; Kang and Petit 1993; Sutherland 1995; Sutherland 1999). As the high viscosity of gellan gum has largely limited the areas for its application, much hope is put on novel enzymes to prepare lower viscosity and low-molecular-mass gellan. Still, the information concerning gellan depolymerizing enzymes and their producers is scared. Two reports on Gram negative bacterial strains capable of producing gellan lyases are known (Kennedy and Sutherland 1994; Sutherland and Kennedy 1996). Gellan lyase synthesis by mesophilic *Bacillus* strains has also recently been described (Hashimoto et al. 1998; Mikolajczak et al. 1994; Miyake et al. 2004).

Thermostable enzymes play an important role in the processes of carbohydrate conversion, where temperatures over 60°C provide high polysaccharide solubility and reduced viscosity of the reaction mixture. To the best of our knowledge, reports on thermostable gellan degrading enzymes are still not available in the literature.

In the present study, we report for the first time biosynthesis of thermostable gellan lyase by thermophilic strain originally isolated from Bulgarian hot spring Sapareva banja.

Materials and methods

Screening

In order to isolate gellan-degrading bacteria, samples from water, soil and algobacterial mats were collected

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from Bulgarian hot springs. One millilitre of each sample was mixed with 9 ml defined medium consisting of 0.1% $(NH_4)_2HPO_4$, 0.02% KCL, 0.01% $MgSO_4$ and 0.2% Gelrite (deacetylated form of gellan, Kelco Division of Merck) as a sole carbon and energy source and cultivated at 60°C, pH 7.5 for 24 h with shaking at 240 rpm. Half a millilitre from those flasks with a registered growth was transferred to 20 ml PY medium (0.2% peptone, 0.1% yeast extract) supplied with 0.2% gellan. Obtained cultures were appropriately diluted, plated on agar PY medium and incubated for 24 h at 60°C for isolation of pure cultures. Visibly different colonies from the samples were selected by formation of pits after growth in a PY medium containing a combination of 1.0% gellan and 0.75% agar (Hashimoto et al. 1997) or by growth in the same medium solidified with 1.5% gellan (Hashimoto et al. 1996). Those cultures, which were able to form pits, indicative of gellan lyase activity, were used for further work.

Staining with Congo red was used as a qualitative method for proving gellan lyase activity. The isolated strains were grown on PY plates, and after 24 h incubation at 60°C, the plates were stained with Congo red (0.1%). The colonies having gellan lyase activity showed a yellowish halo.

Morphology

The morphological and physiological characterization of the chosen strain (98) was performed according to the methods described in "The Genus *Bacillus*" (Gordon et al. 1973) and in "The Genus *Geobacillus*" (Zeigler 2001). Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and partial sequencing of the PCR product (*Escherichia coli* position 20–500) were performed as previously described (Johansen et al. 1999). The partial 16S rDNA sequence was aligned against representative sequences of members of the genus *Bacillus* using the facilities in the ARB program package (Strunk and Ludwig 1995).

Physiological studies

Methods previously described (Smibert and Krieg 1981) were used for physiological characterization of the strain. The determinations of optimal pH and temperature for growth of the chosen strain were performed in the defined medium. Batch fermentation experiments were performed for each pH value in the range 4.5–8.5 (with 0.5 pH steps at 55°C) and each temperature value in the range 50–65°C (with 5°C steps at pH 7.5) on a rotary shaker (240 rpm) in 0.5 l Erlenmayer flasks (three repeats) containing 150 ml medium. An aliquot of 1.5 ml of 20 h preculture was used as an inoculum. Fermentation in a jar fermentor was performed in a 0.5 l fermentor (Model C30, New Brunswick Co., Edison, NY, USA) with working volume 0.35 l. Steady state was assumed after three or more culture volume changes occurred when culture turbidity, pH and gellan lyase activity converged to constant values.

Growth was measured spectrophotometrically on either direct or diluted samples as absorbance at 660 nm against water as a blank.

Analytical methods

Gellan lyase activity was assayed by measuring the reducing sugars in the supernatant according to the dinitrosalicylic acid method (Miller 1959) by using glucose as a standard. The reaction was performed at 70°C and 0.5% gellan solution in 0.02 M Tris–HCl pH 7.0, was used as a substrate. One unit was defined as the amount of enzyme that liberated 1 μM of reducing sugars per minute under the assay conditions.

Lyase activity was also detected spectrophotometrically by continuous increasing of the absorbance at 235 nm based on accumulation of unsaturated glucuronyl end after cleaving of the glycosidic bond between glucosyl and glucuronyl residues in polysaccharide molecule. By such a method the lyase, but not hydrolase mode of polysaccharide degradation was demonstrated. Gellan lyase (1.5 ml of crude enzyme) was added to 1.5 ml of 0.1% gellan solution in 20 mM Tris–HCl buffer, pH 7.0. After incubation at 70°C, the absorbance changes were measured in a Pye Unicam Spectrophotometer at 235 nm. One unit of activity was defined as the amount of enzyme increasing the absorbance by 0.001 unit in 1 h. This assay was used only for determination of the enzyme thermostability.

Lowry's method was used for quantitative determination of protein, using bovine serum albumin as a standard (Lowry et al. 1951).

All data are averages from at least two replicate determinations.

Purification of gellan lyase

Extracellular gellan lyase was purified from the culture liqiud of thermophilic *Bacillus* sp. strain 98.

The culture broth was centrifuged (3,000 *g*, 30 min) to remove the cells. The supernatant was loaded onto an anion-exchange chromatography column (1.6×8.0 cm) containing DEAE-Servacel (Serva), previously equilibrated with 20 mM Tris–buffer (pH 7.0). The column was washed (6 ml/min) with equilibration buffer until no further protein was eluted. Subsequently, bound proteins were stepwise eluted (6 ml/min) with the equilibration buffer containing 0.1 M, 0.4 M and 2.0 M NaCl.

The active fractions were combined, dialyzed against 20 mM Tris–buffer (pH 7.0) for 2 h and concentrated using Millipore standard cassette system with an ultrafiltration membrane (MW cut-off 10 kDa). The concentrate was applied to hydroxylapatite column

(1.6×4 cm) in 10 mM sodium phosphate buffer (pH 6.8). After washing, the proteins were eluted with a linear 10–500 mM gradient of sodium phosphate buffer, pH 6.8.

The active fractions were pooled, concentrated on an ultrafiltration apparatus (Amicon) and loaded on Sephadex G-150 column (2.6×60 cm) equilibrated and eluted with 20 mM Tris–buffer (pH 7.0) containing 0.2 M NaCl. The active fractions were concentrated and further used for electrophoresis.

Protein electrophoresis

SDS-PAGE was performed in 10% resolving gel and 4% stacking gel, essentially as described by Laemmli (14). Samples were heated for 10 min at 100°C. The protein bands were stained with $AgNO_3$. Molecular weights standards 6.5–205 kDa (Sigma) were used.

Results

Isolation of thermophilic gellan lyase producers

One hundred and thirty two environment samples taken from water, soil and algobacterial mat samples of various hot springs areas in Bulgaria were used as inocula for screening and enrichments of aerobic thermophilic producers of gellan lyase. Reducing sugars were registered for 12 of them and two of them with the highest activity (36 and 21 U/l correspondingly) were chosen for isolation of pure cultures. Purification was done by streaking samples on agar at least three times. Single colonies were then subcultured until a pure culture was obtained. The subcultures were considered pure after microscopic observation of one type bacterium per culture. The positive colonies formed crateriform depressions under and around each colony when grown in 1.5% gellan or pits in petri dishes with 1.0% gellan and 0.75% agar. Only colonies which were well separated from others were selected and streaked several times to ensure that a pure culture was obtained.

Morphology and physiology

Between those two isolates, the strain 98 was chosen for further work due to its higher activity (50 U/l). Its colonies were round, whitish, with smooth margins, diameter 3–4 mm. It forms gram-positive, spore-forming non-motile rods 1.68–3.1 μm×0.37–0.53 μm in size. Significant variety in cell morphology was established following 24 h of cultivation, i.e. late stationary phase, observed by electron microscopy. Protoplast forms were observed following 24 h of cultivation. The process of protoplast formation and typical protoplast forms were observed together with bacillary bodies seldom swollen by terminal spores. This strain formed ellipsoidal spores, 0.26–0.8 μm long. It was able to grow in a pH range of 5.5–8.5 at high temperatures (50–82°C). The optimal values for growth determined by their maximal growth were pH 7.0 and 55°C.

The strain is facultative aerobe. The following substrates were found to support growth: glucose, fructose, galactose, ribose, mannose, sucrose, maltose, cellobiose, raffinose, starch, inulin, dextran, glycogen, arabinan, curdlan, galactan, sorbitol, dulcitol, inositol, salicin. The strain was not able to grow on the following substrates: arabinose, rhamnose, xylose, lactose, adonitol, arabinoxylan, cellulose, β-glucan, galactomannan, xyloglucan. Sugars were utilized with formation of acid but no gas. The following tests were positive: nitrate reduction, hydrolysis of casein, production of acid in litmus milk, production of urease. Negative reactions were: growth in 3% NaCl, use of citrate, formation of H_2S, catalase reaction, Voges–Proskauer reaction. Its characteristics are typical for *Bacillus stearothermophilus*, described recently as a representative of a new genus, *Geobacillus stearothermophilus* (Nazina et al. 2001). The affiliation of strain 98 to it was confirmed by partial sequencing of 16S rDNA. Similarity more than 99% with the sequence (X60640) in the ARB database of the type strain of *G. stearothermophilus* was observed.

Optimization of cultivation and gellan lyase production

Activity reached in defined medium was 1.5-fold higher than in PY medium and it was chosen for further work. The optimal concentrations of its components were established to be 0.2% carbon source (gellan) and 0.1% nitrogen source $(NH_4)_2HPO_4$. Time courses of a growth and gellan lyase synthesis of the strain in that medium are presented in Fig. 1.

The enzyme was secreted extracellularly and no intacellular activity was detected in both, exponential (14 h) and stationary (28 h) phase. It was produced

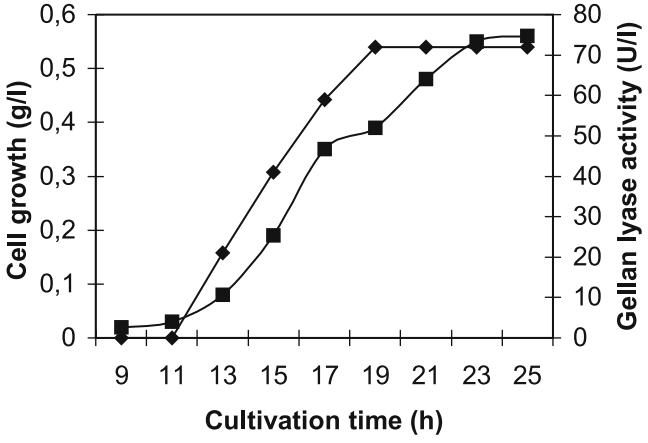

Fig. 1 Time course of growth and gellan lyase synthesis from *G. stearothermophilus* at 55°C, pH 7.5, 240 rpm in a defined medium. Samples were taken in 1 h interval and assayed for growth (*line with square*) and gellan lyase activity (*line with diamond*)

during an exponential phase of growth. The activity was registered only in the culture medium, Comparatively long lag-phase was observed in the medium used. Maximum gellan lyase activity was reached after 20 h of cultivation at the end of exponential phase. The shortest generation time of 60 min and μ_{max} of 0.69 h^{-1} were established at pH 7.0, and enzyme concentration was highest at pH 7.5. Similar doubling time (56 min) was registered after growth in PY medium. Optimal temperature for growth and enzyme synthesis was 55°C. The viscosity of the culture decreased simultaneously with increasing cell growth and reached water viscosity in the stationary phase indicating carbohydrate utilization for growth. No growth was observed in defined media containing xanthan or starch as the sole carbon source and growth but no activity was registered in PY medium in which gellan was replaced by the above carbohydrates. Batch cultures in a fermentor reached similar activity as those in flasks (79 U/l).

Continuous cultivation

Figure 2 illustrates the effect of dilution rate on the volumetric productivity of gellan lyase. Enzyme activity was dependent of dilution rate and its maximum occurred at low dilution rate of 0.09–0.13 h^{-1}. Activity registered at this rate (700–720 U/l) was more than tenfold higher in comparison with batch cultivation. Increase of the dilution rate from 0.13 to 0.3 h^{-1} caused a drastic drop in enzyme production although optical density of the culture remained almost constant. Enzyme productivity reached in continuous cultures at D 0.13 was 66.0 U/l/h.

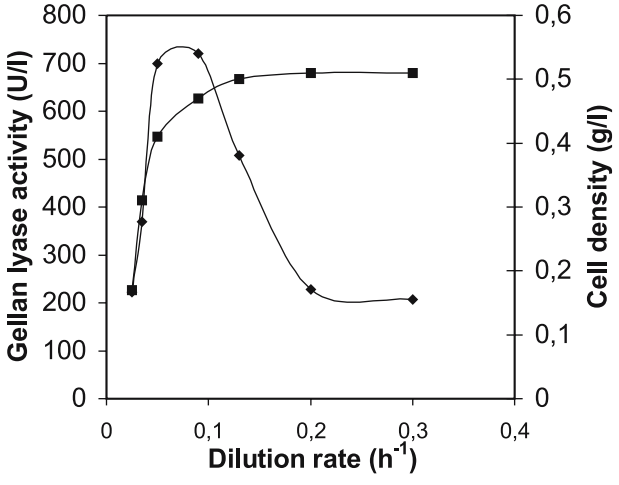

Fig. 2 Growth (*line with square*) and gellan lyase activity (*line with diamond*) in continuous cultures of *G. stearothermophilus* 98 in MSM at 55°C, pH 7.5, 300 rpm and aeration 1.0 vvm (volume air per volume medium per minute)

Enzyme purification

The purification procedure is summarized in Table 1. It comprises ion exchange chromatography on DEAE Servacell, hydroxylapatite chromatography and gel-chromatography on Sephadex G-150. As a result, 40.5% yield was reached and the protein was purified ninefold. The relatively not very high yield and purification factor suggest an easy aggregation between enzyme molecules followed by their inactivation. Similar aggregation was observed in the process of enzyme preserving at 4°C, and it was overcome by adding 5% DMSO. Purified enzyme was eluted very closely to the void volume on Sephadex G-150. In SDS gel the protein showed a molecular weight at about 120,000 Da and two protein bands were observed in hydoxylapatite fraction in native electrophoresis gel—one with a molecular weight at about 480,000 Da and another at about 350,000 Da, suggesting the multimeric nature of the enzyme, a fact often observed for thermostable enzymes.

Physico-chemical properties of the purified gellan lyase

The effect of temperature on gellan lyase activity was studied from 40 to 80°C at pH 7.0 and was very similar to that for unpurified enzyme. The enzyme was active in the range of 50–80°C with temperature optimum at 70°C and differs significantly from the temperature optima (30–45°C) described for other gellan lyases (Hashimoto et al. 1999; Kennedy and Sutherland 1994). Its activity at 55°C was 50% of the activity at 70°C. The enzyme expressed also unusual high thermostability (Fig. 3). It retained 100% of its activity after being heated at 60°C for 24 h. Its half life at 70°C was 50 min in absence of substrate. The presence of the substrate additionally stabilizes the enzyme and its residual activity after 2.5 h at 70°C was 100%.

Table 1 Purification steps of *Bacillus stearothermophilus* gellan lyase

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Yield %	Purifi-cation factor
Cell free broth	2,000	0.27	540	0.03	60	0.11	100	1
DEAE-servacell	400	0.125	50	0.09	36	0.72	60	6.5
Hydroxylapatite	30	0.954	28.62	0,887	26.61	0.93	44.35	8.5
Sephadex G-150	30	0.82	24.18	0.81	24.3	0.99	40.5	9.0

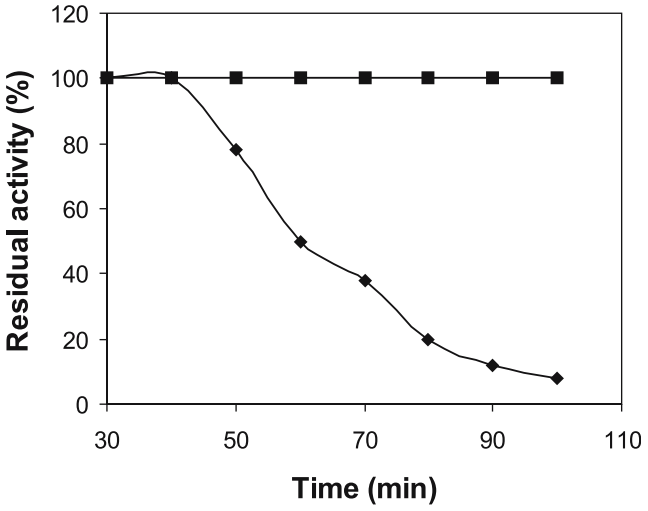

Fig. 3 Thermostability of *G. stearothermophilus* gellan lyase in presence (*line with square*) and absence (*line with diamond*) of gellan. The enzyme solution (700 U/l determined by DNS method, 0.5% gellan as a substrate and 97 U/l determined spectrophotometrically at 235 nm, 0.1% gellan as a substrate) was preincubated at 70°C for various timings. Residual activity was analyzed spectrophotometrically by detection of increasing absorbance at 235 nm

Table 2 Effect of some ions and inhibitors on gellan lyase activity

Compound	Relative activity, %
Metal ions	
Controlle	100
$ZnSO_4 \cdot 7H_2O$	100
KCl	136.7
Li_2SO_4	130
$CoCl_2$	110
NaCl	117.7
$CaCl_2$	136.7
$FeCl_3 \cdot 6H_2O$	113.3
$FeCl_2$	120
$MnCl_2 \cdot 4H_2O$	120
$MgCl_2 \cdot 6H_2O$	123
$HgCl_2$	106.7
$CuSO_4 \cdot 5H_2O$	50
Inhibitors	
Urea	77.5
EDTA	77.5
Dithiotreitol	87.1
p-CMB	61.3
PSF	67.8
Natrium lauryl sulfate	19.35
N-bromosuccinimide	19.35

The enzyme was preincubated for 30 min at 60°C in Tris–HCl buffer, pH 7.0, containing different ions and inhibitors at final concentration 1 mM and the activity was then determined at 70°C, pH 7.0

The enzyme activity was studied at pH values from 4.0 to 10.0. The enzyme was the most active in very large pH area (5.0–8.0).

The effect of various compounds on gellan lyase activity was established (Table 2). In the presence of a number metal ions in a low concentration (1 mM) a stimulating effect on activity was observed for almost all ions tested. Only Cu^{2+} inhibited enzyme activity, suggesting tryptophan presence in the active site. Metal ions in high concentration (1 M) inhibited enzyme activity. The residual activity in presence of K^+, Na^+ and Mg^{2+} at such a concentration was no more than 10%. The enzyme was sensitive to all inhibitors used with highest degree of inhibition toward N-bromosuccinimide, indicating the important role of tryptophan (histidine) in the active center of the enzyme.

Discussion

To the best of our knowledge, the newly isolated strain of *G. stearothermophilus* is the first reported thermophilic producer of thermostable gellan lyase. It has been found that enzyme degrade deacylated gellan due to the extracellular eliminase type of enzyme (lyase), which cleave the sequence –β-D-glucosyl-(1 → 4)- –β-D-glucuronosyl- in the tetrasaccharide repeat unit. It is an endoenzyme and a significant loss of solution viscosity was observed after treatment of 0.2% solution with the purified enzyme (data not shown). The gellan lyases are newly identified in a series of bacterial exopolysaccharides, such alginate lyases, xanthan lyase, heparinase and chondroitinase. These enzymes cleave the main chain of uronic acid containing polysaccharide substrates. Although other members of sphingan family were not examined as substrates due to their absence in our lab, the failure of the enzyme to hydrolyze other structurally related exopolysaccharides like xanthan and alginate may, therefore, indicate its high substrate specificity. The unusual substrate specificity of gellan-degrading enzymes even to other sphingans was noticed previously (Mikolajczak et al. 1994). Unlike *Pseudomonas aeruginosa* and *Sphingomonas* strains able to produce corresponding alginate lyase and gellan lyase but unable to utilize alginate or gellan as the sole carbon source (Sutherland and Kennedy 1996), *G. stearothermophilus* 98 was able to grow in a mineral medium with gellan as the only carbon and energy source. Gellan lyase synthesis was expressed only in the presence of gellan in the medium and growth but no activity was registered in PY medium in which gellan was replaced by other carbohydrates. Its strongly inducible synthesis was similar to some gram-negative bacteria (Kennedy and Sutherland 1994) and differs from constitutive gellanase synthesis detected in *Sphingomonas* strains (Sutherland and Kennedy 1996). Unlike the enzyme from *G. stearothermophilus* 98, the enzymes secreted by *Bacillus* sp. GL1 was induced not only by gellan but also by xanthan (Hashimoto et al. 1998).

Productivity reached in continuous cultures at D 0.13 (66.0 U/l/h) was almost sixfold higher in comparison with batch cultures (11.7 U/l/h). These results clearly demonstrate that overproduction of the enzyme can be achieved in continuous cultures and they are in good agreement with the results achieved by other authors for

overproduction of thermostable extracellular enzymes (Becker et al. 1997; Emanuilova et al. 1999).

The enzyme synthesized by *G. stearothermophilus* 98 is the first reported thermostable gellan lyase, whose unusual high thermostability is incomparable with previously described enzymes. Residual activities of other enzymes (Hashimoto et al. 1994; Hashimoto et al. 1996) were 100% after 10 min treatment with temperatures up to 40°C. Having in mind that gellan is resolved at temperatures above 60°C, the indicated thermostability determines the enzyme as an ideal candidate for application in the biotechnological processes. The large pH area for its optimal action and a lack of inhibition in many metal ions in low concentration suggests a possible successful application of the enzyme in different industrial conditions.

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