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Extracellular deoxyribonuclease production by a thermophile, *Streptomyces thermonitrificans*

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A thermophilic bacterial strain, *Streptomyces thermonitrificans*, produced high levels of extracellular deoxyribonuclease (DNase) when grown on NBG medium (containing 1% peptone, 0.3% beef extract, 1% glucose and 0.5% NaCl). Maximum DNase activity (140 U ml^{-1}) was obtained, in 24 h, when the culture was grown on modified NBG medium (containing 1.3% beef extract, 1% glucose, 0.5% NaCl and 50 μM Mn^{2+} at 45°C. The crude enzyme showed higher activity on native DNA than on sonicated and heat denatured DNA. Moreover, addition of Mn^{2+} in the assay mixture resulted in a significant stimulation (10–15 fold) of the enzyme activity.

Keywords: *Streptomyces thermonitrificans*; thermophilic *Streptomyces* sp; extracellular DNase; enzyme production; metal ions

Introduction

Nucleases are important analytical enzymes and have extensive applications in the determination of nucleic acid structure [9,13]. Though they are widely distributed, the enzymes of analytical interest originate mainly from microbial sources. A majority of the enzymes reported so far require metal ions for their activity, which in turn prevents their use in chelating buffers or in the presence of metal chelators. Hence, there is a need for a different type of nuclease which either does not require metal ions for its activity or can exhibit sufficient activity in the absence of metal ions. Members of the genus *Streptomyces*, by their ability to degrade various biopolymers, are known to produce a wide variety of extracellular hydrolases [12]. Though they are extensively studied for the characterization of restriction endonucleases, very little information is available on DNases from these organisms. Moreover, most of the strains of *Streptomyces* reported so far are mesophilic and no report exists on thermophilic organisms. Since thermophilic organisms grow at high temperatures, their use not only helps to prevent microbial contamination but also permits optimum enzyme production in a shorter time than mesophilic organisms. In view of this, several *Streptomyces* cultures from the National Collection of Industrial Microorganisms (NCIM) were screened to evaluate their extracellular nuclease producing ability. Among them, *Streptomyces thermonitrificans*, which exhibited high extracellular DNase activity at pH 7.0 and 45°C, was selected for optimization studies and the results are presented in this communication.

Materials and methods

Materials

Beef extract (HiMedia Laboratories Pvt Ltd, Bombay, India; Loba Chemie, Bombay, India; Qualigens Fine Chemicals, Bombay, India; and Difco Laboratories, Detroit, MI, USA); tryptone, yeast extract, peptone and casamino acids (Difco Laboratories) were used. All other chemicals were of analytical grade. High molecular weight DNA from buffalo liver was prepared according to Mehra and Ranjekar [10].

Microorganism and growth

The thermophilic strain of *S. thermonitrificans* obtained from the National Collection of Industrial Microorganisms (NCIM 2007), National Chemical Laboratory, Pune, India, was maintained at 45°C on MGYP slants (malt extract 0.3%; yeast extract 0.3%; peptone 0.5%; glucose 1.0% and agar 2.0%).

Enzyme production

The inoculum was prepared by inoculating 10 ml of NBG medium (g L^{-1}: beef extract, 3; peptone, 10; glucose, 10 and NaCl, 5; adjusted to pH 7.0) with a 4-day-old well-sporulated piece of agar slant, followed by incubation at 45°C for 32 h with shaking (200 rpm). Optimization studies were carried out in 250-ml conical flasks, containing 50 ml of medium, using inoculum medium as the control by transferring 10% inoculum followed by incubation at 45°C for 24 h with shaking (200 rpm). The extracellular broth was collected by centrifugation (9000 × *g*, 20 min) and used as the source of enzyme. All experiments were performed in duplicate.

DNase assay

The assay for DNase activity was carried out essentially according to Gite *et al* [8]. The standard reaction mixture of 1.0 ml contained 50 μg of native or sonicated and heat denatured DNA in 30 mM Tris-HCl buffer, pH 7.0 and appropriately diluted enzyme. The reaction was initiated by the addition of DNA followed by incubation at 37°C for 15 min. The reaction was then terminated by adding 1 ml of 10% (v/v) chilled perchloric acid and 1 ml of 0.2% (w/v) BSA. The mixture was left on ice for 10 min and then centrifuged (2000 × *g*, 20 min) to sediment the precipitate. The

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acid soluble nucleotides in the supernatant were measured at 260 nm. The acid soluble deoxyribonucleotides were estimated by assuming a molar absorption coefficient of 10 000 M^{-1} cm^{-1} [5].

One unit of DNase activity is defined as the amount of enzyme required to liberate 1 μmol of acid-soluble nucleotides min^{-1} under the assay conditions.

Determination of intracellular DNase activity

The cells were harvested by centrifugation (9000 × *g*, 20 min), washed with distilled water and suspended in 30 mM Tris-HCl buffer, pH 7.0 (1 g wet weight in 5 ml buffer). The cells were sonicated using a Ralsonic sonicator at 10 000 Kcyc for 6 min and the cell debris was removed by centrifugation (9000 × *g*, 20 min). The supernatant was used for determining enzyme activity.

Determination of protein

Protein was estimated according to Sedmak and Grossberg [14] using BSA as standard.

Determination of reducing sugar

Reducing sugar was determined by the DNSA method of Miller [11].

Estimation of metal ion content of beef extracts

The magnesium, manganese and calcium contents of different beef extract samples were determined by atomic adsorption on a ATI UNICAM 929 AA spectrophotometer at 285.10 nm, 279.48 nm and 422.7 nm respectively.

Results and discussion

Effect of carbon sources

Streptomyces thermonitrificans produced high levels of extracellular DNase when grown in NBG medium containing 1% (w/v) glucose. All the carbon sources tested including fructose, maltose, lactose, sucrose, soluble starch and glucose, supported growth as well as enzyme production. However, maximum DNase activity was obtained in the presence of glucose. Tests on the influence of glucose concentration (0.5–2.0% w/v) on enzyme production revealed that maximum enzyme levels (10 U ml^{-1}) were obtained in the presence of 1% (w/v) glucose (Table 1). An increase in glucose concentration (>1% w/v) inhibited growth as well as enzyme production. In *Neurospora crassa*, it was shown that sorbose enhances extracellular DNase levels [7]. In the present case, sorbose when used as the sole carbon source, supported growth but did not enhance enzyme production. Similarly, in the case of *Streptomyces levoris*, soluble starch enhanced extracellular DNase levels [3] but in *S. thermonitrificans*, though it supported growth, it had no effect on the enzyme production.

Table 1 Effect of carbon sources on extracellular DNase production by *S. thermonitrificans*

Carbon source[a] (1% w/v)	dsDNase activity (U ml^{-1})	pH
Glucose	10.0	8.3
Sucrose	7.6	8.5
Fructose	3.6	8.5
Mannose	3.6	8.4
Maltose	4.0	8.5
Lactose	2.0	8.6
Soluble starch	5.6	8.4

[a]Grown on NBG medium (without glucose) for 24 h, with the carbon sources indicated.

Effect of nitrogen sources

In *S. levoris*, inorganic nitrogen salts *viz* ammonium nitrate and ammonium chloride were reported to be the best sources of nitrogen [2]. In the present study, the influence of inorganic nitrogen sources on enzyme production was studied using Czapek-Dox medium without a nitrogen source and with glucose as the sole carbon source (instead of sucrose) as the basal medium. However, inorganic nitrogen sources did not support growth of *S. thermonitrificans.* Apte *et al* [1] while studying the influence of various organic nitrogen sources on extracellular DNase production by *Rhizopus stolonifer*, noted that among the various peptones tried, only one, ie the one obtained from Sarabhai M Chemicals, Baroda, India, supported growth and enzyme production. In the present studies, among the various organic nitrogen sources tested, namely, yeast extract, tryptone, peptone, casamino acids, urea and beef extract, only beef extract resulted in higher enzyme levels. Interestingly, the type of beef extract used had a marked effect on growth and enzyme production. Among them, the beef extract obtained from HiMedia Laboratories, Bombay, India, gave maximum enzyme yield. Maximum DNase levels (53 U ml^{-1}) were obtained when beef extract was used at a concentration of 1.3% (w/v) (Table 2). Above this level a slight depression of enzyme activity was observed.

Effect of metal ions

In *Actinomyces cinerofuscus* divalent metal ions like Mg^{2+} and Ca^{2+} are required for optimal DNase production [15]. Though *S. thermonitrificans* could grow and produce enzyme in the absence of added metal ions, higher enzyme levels were obtained in the presence of 50 μM Mn^{2+}. To ascertain the probable cause for the difference in DNase levels with different beef extracts from different manufac-

Table 2 Effect of different beef extracts and metal ions on extracellular DNase production by *S. thermonitrificans*

Beef extract (at 1.3% w/v)	dsDNase activity (U ml^{-1})	Final pH
Control[a]	10.0	8.3
Difco	ND	5.8
Difco + Mn^{2+} (50 μM)	ND	6.3
HiMedia	53.0	8.4
HiMedia + Mn^{2+} (50 μM)	140.0	8.4
Loba	2.0	7.7
Loba + Mn^{2+} (50 μM)	27.3	7.9
Qualigens	2.0	6.9
Qualigens + Mn^{2+} (50 μM)	45.5	7.5

ND, not detected.
[a]Grown on basal NBG medium containing beef extract 0.3% w/v, Difco peptone 1% w/v, glucose 1% w/v and NaCl 0.5% w/v, for 24 h.

Table 3 Metal content of different beef extracts

Component[a]	Source			
	Difco	Loba Chemie	HiMedia	Qualigens
Mg^{2+}	0.91	30.5	31.75	27.54
Mn^{2+}	0.82	0.145	1.0	0.15
Ca^{2+}	4.15	310.8	127.8	43.81

[a]The above values represent the effective concentrations (metal ions in ppm) of each component when the respective beef extracts were used at a concentration of 13.0 g L^{-1} of the medium.

turers, the metal ion content of each beef extract was determined (Table 3). To evaluate the role of different metal ions, Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+} and Ca^{2+}, in the production of extracellular DNase, each of these metal ions was added to the medium and enzyme yield was monitored. Modified NBG medium (1.3% w/v HiMedia beef extract, 1% w/v glucose and 0.5% w/v NaCl) served as a control medium. Addition of 50 μM of Co^{2+}, Cu^{2+} or Zn^{2+} inhibited growth while Fe^{2+} and Ca^{2+} supported growth but not enzyme production. Addition of Mg^{2+} showed a marginal increase while Mn^{2+} brought about approximately a 3-fold increase in enzyme levels (Table 4). Further increase in Mn^{2+} concentration did not enhance the enzyme activity (data not shown). Addition of Mn^{2+} in the NBG medium containing Loba and Qualigens beef extract showed approximately 14- and 22-fold increases in enzyme levels. However, addition of Mn^{2+} in the NBG medium containing Difco beef extract did not show any increase in enzyme levels (Table 2).

Effect of initial pH and temperature

S. thermonitrificans was able to grow and produce the enzyme only over a narrow pH range, 7.0–9.0, and failed to grow outside this range. However, maximum enzyme levels were obtained when the initial pH of the medium was adjusted to 7.0 (Table 5). Tests on the influence of temperature on extracellular DNase production revealed that though *S. thermonitrificans* could grow and produce the enzyme between 37 and 55°C, maximum enzyme activity (140 U ml^{-1}) was obtained when the culture was grown at 45°C.

Table 4 Effect of different metal ions on extracellular DNase production by *S. thermonitrificans*

Metal ions	dsDNase activity (U ml^{-1})	pH
Control (C)	53.0	8.3
C + Mg^{2+}	82.1	8.1
C + Mn^{2+}	140.0	8.4
C + Fe^{2+}	0.0	8.2
C + Cu^{2+}	ND	–
C + Zn^{2+}	ND	–
C + Ca^{2+}	ND	8.3
C + Co^{2+}	ND	–

ND, not detected.
C, grown on modified NBG medium containing 1.3% (w/v) Hi-Media beef extract, glucose 1% (w/v) and NaCl 0.5% (w/v) for 24 h and metal ions as indicated above.

Table 5 Effect of initial pH on extracellular DNase production by *S. thermonitrificans*

Initial pH of the medium	dsDNase activity (U ml^{-1})	Final pH
7.0	53.3	8.1
8.0	30.0	8.3
9.0	18.0	8.4

Profile of growth and enzyme production

The time course of cultivation of *S. thermonitrificans* in the production medium is shown in Figure 1. The pH of the medium gradually increased and at optimal enzyme levels the pH of the medium was above 8.0. A similar increase in the pH of the medium was observed in *Streptomyces* sp during glucose isomerase production [4,6]. Extracellular DNase production was maximum at 24 h. Concomitantly, intracellular DNase levels also increased until maximum reducing sugar was utilized (32 h) and then it decreased.

Effect of metal ions on enzyme activity

Baskakova *et al* [2] observed that DNase from *Actinomyces* sp is activated by divalent cations like Mn^{2+}, Mg^{2+} and Co^{2+}. This is also true in the case of *S. thermonitrificans*, as the inclusion of 2 mM (effective concentration) of either

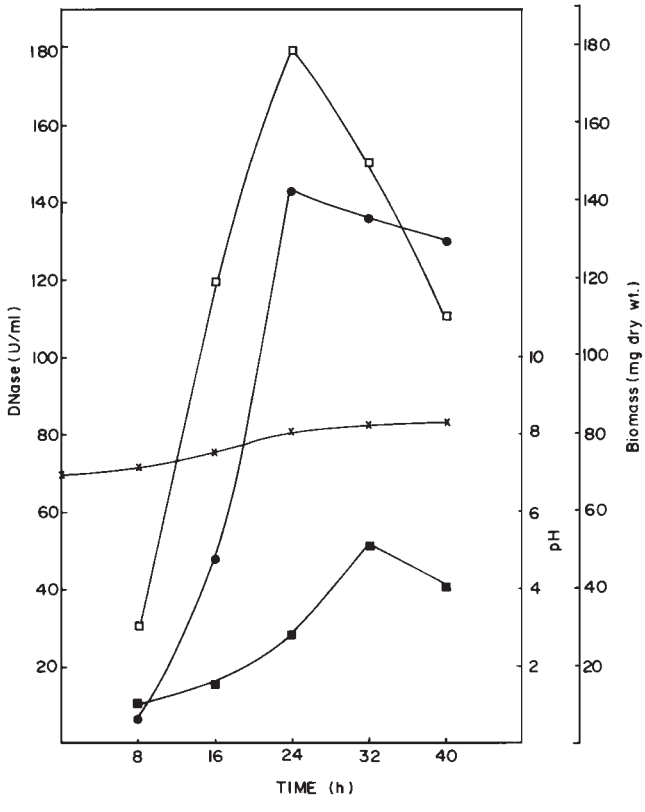

Figure 1 Profile of growth and DNase production by *Streptomyces thermonitrificans* in submerged culture: extracellular DNase activity (●), pH (X) and intracellular DNase activity (■), biomass (□).

Table 6 Effect of metal ions on DNase activity

Metal salt	Concentration (mM)	dsDNase activity (U ml^{-1})
Control[a]	–	63
$MgCl_2$	2.0	153
$CoCl_2$	2.0	266
$MnCl_2$	2.0	870

[a]Extracellular broth obtained from modified NBG medium, in the absence of Mn^{2+}, was used as control.

Mg^{2+}, Co^{2+} or Mn^{2+} in the assay mixture enhanced enzyme activity. While the addition of Mg^{2+} or Co^{2+} brought about a 3- to 4-fold increase in the DNase activity, considerable stimulation of the activity (10–15 fold) was observed in the presence of Mn^{2+} (Table 6).

Conclusion

S. thermonitrificans produces high activity of extracellular DNase (10 U ml^{-1}) in NBG medium (pH 7.0) at 45°C in 24 h. Higher activity (53 U ml^{-1}) was obtained when peptone in the NBG medium was replaced by beef extract. Moreover, addition of a low concentration of Mn^{2+} (50 μM) in the production medium brought about an approximately 3-fold increase in enzyme levels (140 U ml^{-1}). Most of the analytically important nucleases show an obligate requirement for divalent cations for their activity and this prevents their use in chelating buffers or in the presence of metal chelators. In this respect, *S. thermonitrificans* DNase has the advantage in that, though the addition of Mn^{2+} in the assay mixture significantly enhances the enzyme activity (10–15 fold), high activity (53 U ml^{-1}) can also be obtained in the absence of added metal ion. The crude enzyme exhibited higher activity on native DNA suggesting that it prefers double stranded DNA as substrate. However, extensive purification and specificity studies are required to support this view.

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References

1 Apte PS, MV Deshpande and V Shankar. 1993. Optimization of medium for extracellular nuclease formation from *Rhizopus stolonifer*. World J Microbiol Biotechnol 9: 205–209.
2 Baskakova AA, AV Baldina and AM Bezborodov. 1967. Some properties of deoxyribonuclease from *Actinomycetes*. Mikrobiologiya 36: 19–23. (Chem Abstr 66: 102043w).
3 Bezborodov AM, AA Baskakova and AV Baldina. 1967. Conditions of *Actinomyces* (*Streptomyces*) *levoris* 2789 deoxyribonuclease biosynthesis. Mikrobiologiya 36: 390–395. (Chem Abstr 67: 51325a).
4 Chen WP, AW Anderson and YW Han. 1979. Production of glucose isomerase by *Streptomyces flavogriseus*. Appl Environ Microbiol 37: 324–331.
5 Curtis PJ, MG Burdon and RMS Smellie. 1966. The purification from rat liver of a nuclease hydrolyzing ribonucleic acid and deoxyribonucleic acid. Biochem J 98: 813–817.
6 Deshmukh SS, MV Deshpande and V Shankar. 1994. Medium optimization for the production of glucose isomerase from thermophilic *Streptomyces thermonitrificans*. World J Microbiol Biotechnol 10: 264–267.
7 Fraser MJ. 1979. Alkaline deoxyribonucleases released from *Neurospora crassa* mycelia: two activities not released by mutants with multiple sensitivities to mutagens. Nucleic Acids Res 6: 231–246.
8 Gite S, G Reddy and V Shankar. 1992. Active site characterization of SI nuclease: affinity purification and influence of amino group modification. Biochem J 285: 489–494.
9 Gite SU and V Shankar. 1995. Single-strand-specific nucleases. Crit Rev Microbiol 21: 101–122.
10 Mehra U and PK Ranjekar. 1979. Buffalo (*Bos bubalus*) genome: occurrence and characterization of repeated DNA sequences. Ind J Biochem Biophys 16: 56–60.
11 Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31: 426–428.
12 Peczynska-Czoch W and M Mordarski. 1988. Actinomycetes enzymes. In: Actinomycetes in Biotechnology (Goodfellow M, ST Williams and M Mordarski, eds), pp 219–283, Academic Press, San Diego.
13 Reddy LG and V Shankar. 1993. Immobilized nucleases. Crit Rev Biotechnol 13: 255–273.
14 Sedmak JJ and SE Grossberg. 1977. A rapid, sensitive and versatile assay for protein using Coomassie Brilliant Blue G250. Anal Biochem 79: 544–552.
15 Verzilov VV, VN Maksimov and NA Krasilnikov. 1972. Optimum growth media for the production of nucleases by *Actinomycetes*. Mikrobiologiya 41: 964–972. (Chem Abstr 78: 134474b).