

1-1-2009

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AYGAN, ASHABIL and ARIKAN, BURHAN (2009) "Production and characterization of multifunctional endoxylanase by *Bacillus* sp. X13," *Turkish Journal of Biology*. Vol. 33: No. 3, Article 8. <https://doi.org/10.3906/biy-0806-18>

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Production and characterization of multifunctional endoxylanase by *Bacillus* sp. X13

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Received: 17.06.2008

Abstract: A multifunctional endoxylanase producing *Bacillus* sp. was isolated from soil. Enzyme synthesis occurred at temperatures between 20 °C and 60 °C with an optimum 37 °C. Analysis of the enzyme by SDS-PAGE revealed 4 active enzyme bands, which were estimated to be 66.5 kDa, 80.6 kDa, 95.5 kDa, and 108.4 kDa. The enzyme has a broad temperature range, between 20 and 90 °C, with an optimum at 40 °C; and maximum activity was at pH 6.0. The enzyme showed a gradual decrease in the remaining activity as the pre-incubation temperature increase. Thermostability was not also increased in the presence of Ca²⁺. An average of 71% of remaining activity observed when the enzyme incubated between pH 3.6 and 10 for 1 h. The enzyme was highly inhibited by urea, and fairly inhibited by Triton X-100, CaCl₂, ZnCl₂, KCl, Na₂SO₃, PMSF, and 2-Mercaptoethanol. The properties of the enzyme presented in this study suggest that this enzyme could be a potential industrial interest.

Key words: *Bacillus* sp., extracellular, endoxylanase, xylanase, multifunctional

Bacillus sp. X13 suşundan multifonksiyonel endoksilanaz üretimi ve karakterizasyonu

Özet: Toprakta multifonksiyonel endoksilanaz üreten *Bacillus* sp. X13 suşu izole edilmiştir. Enzim sentezi optimum 37 °C olmak üzere 20 ile 60 °C arasında gerçekleşmiştir. SDS-PAGE analizinde, 66,5 kDa, 80,6 kDa, 95,5 kDa ve 108,4 kDa olarak hesaplanan 4 aktif enzim bandı gözlenmiştir. Enzim optimum aktivitesi 40 °C olup 20-90 °C arasında geniş bir sıcaklık aralığına sahiptir ve maksimum aktivitesi ise pH 6,0 olarak bulunmuştur. İnkübasyon sıcaklığı arttıkça enzim kalan aktivitede giderek artan bir düşüş göstermiştir. Sıcaklık stabilitesi Ca⁺² varlığında dahi artmamıştır. Enzim pH 3,6 ile 10,0 arasında 1 saat inkübe edildiğinde kalan aktivite ortalama % 71 olarak elde edilmiştir. Enzim üre ile yüksek oranda inhibe olurken Triton X-100, CaCl₂, ZnCl₂, KCl, Na₂SO₃, PMSF ve 2-Merkaptoetanol varlığında çok düşük oranda etkilenmiştir. Bu çalışmada enzimin belirlenen özellikleri bu enzimin endüstriyel kullanımında potansiyel bir tercih sebebi olabilir.

Anahtar sözcükler: *Bacillus* sp., ekstraselüler, endoksilanaz, ksilanaz, multifonksiyonel

Introduction

Xylan is a major component of plantal cell walls. It is a complex molecule composed of β -1,4-linked xylose chains with branches of neutral or uronic mono and oligosaccharides. Biodegradation of xylan entails the action of many enzymes. Among them, endo-1,4- β -xylan xylanohydrolase (EC 3.2.1.8) plays a key role by degrading the xylan backbone into small oligomers (1,2). As a source of enzyme, microbial xylanases are more advantageous over their counterparts from plant and animal sources given the easy production capacity, structural stability, and convenience of genetic applications (3). The main constituents of microbial xylanolytic enzyme systems are endoxylanase (Endo-1,4- β -xylan xylanohydrolase) and b-xylosidase (1,4- β -D-Xylan Xylohydrolase) (4). In terms of industrial applications, xylanases are used in food, pharmaceuticals, paper-pulp, agricultural waste processing, and feed conversion efficiency (1,5,6). Members of the genus *Bacillus* produce different xylanases and this genus are used extensively in industrial fermentation, since they secrete most of their enzymes into the extracellular medium. Xylanases from various fungi and bacteria have been isolated. Bacterial xylanases range from acidic to alkaline depending on the type of the organism, unlikely fungal xylanases that are often naturally acidic to neutral. Most organisms produce multiple forms of xylanases as a result of differential mRNA processing and posttranslational modifications, as well as the product of multiple genes (7). Although xylanase activities reported from some fungal strains (8, 9), the presence of considerable amount of cellulase activity make the enzyme unsuitable for pulp and paper industries since degradation of cellulose is the main problem in pulping processes. On the other hand, multifunctional enzymes are important in clarifying juices and wines, extracting coffee, plant oils and starches for increasing nutritional value of agricultural silage, and grain feed (10). Additionally, this kind of enzymes in bioconversion of lignocelluloses to fermentable sugars from agrowaste has the economical prospect to desirable hydrolysis. Therefore, it is important to find novel microorganisms that can produce suitable xylanases for environmental and industrial applications.

In this paper, we report the isolation of *Bacillus* sp. X13 from soil and partial purification and the

characterization of multifunctional endoxylanase from it. The properties of the enzyme presented here suggest that this enzyme could be a potential choice for industrial interest.

Materials and methods

Organisms and cultivation conditions

Bacillus sp. X13 was isolated from soil samples collected from Tarsus, Turkey. This study selected gram-positive spores forming bacteria, *Bacillus* sp., by pasteurizing samples at 80 °C for 10 min. A total 242 bacterial isolates were screened for xylanase production on agar plates containing: peptone, 5 g/L; yeast extract, 1 g/L; NaCl, 5 g/L; MgSO₄ x 7H₂O, 0.2 g/L; CaCl₂, 0.1 g/L; K₂HPO₄, 1 g/L; Xylan (Oat Spelt), 5 g/L; Agar agar, 15 g/L. The initial pH was adjusted to 7.0 after autoclaving with 1 N HCl (11). A total of 32 (13.2%) xylanolytic isolates were selected by flooding the agar plates with Congo Red solution (0.1%) (12). The xylanolytic potential was estimated using the xylanolytic ratio (R/r), defined as the diameter of the hydrolyzation zone (R) divided by the diameter of the producing colony (r) (13).

Enzyme production

The strain *Bacillus* sp. X13 was fermented in the medium containing: Xylan (Oat spelt), 5 g/L; peptone, 5 g/L; yeast extract, 1 g/L; NaCl, 5 g/L; K₂HPO₄, 1 g/L; MgSO₄, 0.2 g/L; CaCl₂, 0.1 g/L. Cultures were grown for 24 h at 37 °C with shaking at 200 rpm. After the removal of cells by centrifugation (Hettich Universal 30 RF) (11,200 g, 20 min) at +4 °C, the supernatant was used for further work (11).

Partial purification of xylanase

The clear supernatant was concentrated with ethanol previously chilled by adding dropwise at 4 °C with continuous stirring, and solution was left at -30 °C for 24 h. The enzyme was recovered by re-suspending the precipitate in 100 mM with phosphate buffer at pH 7.2 (14).

Enzyme assay

Xylanase activity was assayed by adding 0.5 mL of enzyme to 0.5 mL of xylan (1% v/v) in 100 mM phosphate buffer, pH 6.0, and incubating at 40 °C for 30 min. The reaction was stopped by the addition of 1 mL of 3,5-dinitrosalicylic acid reagent and

absorbance was measured in a Cecil 5,500 spectrophotometer (A_{550}). One unit of enzyme activity was defined as the amount of enzyme releasing 1 mmol of reducing sugars per minute under the standard assay conditions.

Effect of pH and temperature on activity and stability

The effects of temperature and pH on enzyme activity were examined at different temperatures ranging from 4 to 100 °C and at pH values from 4.0 to 11.0 for 30 min. The following buffers were used in the reactions: 100 mM Na-Acetate Acetic acid (pH 4.0-5.0), 100 mM Na-Phosphate buffer (pH 6.0-7.0), 100 mM Tris-HCl buffer (pH 8.0-10.0), and 100 mM Borax-NaOH buffer (pH 11.0). To determine the temperature stability, the enzyme was pre-incubated between temperatures of 20 and 90 °C for 15 and 60 min at the optimum pH, the remaining activity was determined under standard enzyme conditions. For determining the stability of pH, the enzyme was pre-incubated at pH's ranged from 3.6 to 10.0 at 40 °C for 15 and 60 min, 24 h, and then measured the remaining activity at standard enzyme conditions. The activity of the enzyme stored at +4 °C was used as a control for thermal stability, pH stability, and other assays. The experiments were repeated three times and the mean values were taken.

Effects of metal ions and chemical agents on enzyme activity

The effect of metal ions and some chemicals, including chelating agents, and inhibitors on xylanolytic activity was studied by pre-incubating the enzyme in the presence of substances with a final concentration of 3 or 5 mM for 15 and 30 min at 40 °C, and then performing the assay in the presence of the same substances at the optimum temperature (15). All metals used were in the chloride form. The activity in the absence of any additives was taken to be 100%.

PAGE analysis and activity staining

SDS-PAGE (10%) containing xylan (1%) (16) was carried out to determine molecular mass. After electrophoresis, the gel was cut into two pieces, one was used for staining, and the other was subjected to renaturation solutions. The protein bands were detected by destaining the gel in a methanol-acetic acid-water solution (4:1:5 by volume) after a staining

process with 0.1% Coomassie Blue R 250 (17,18). Activity staining was performed after incubating the gel in 0.1% Congo Red solution for 1 h (19). The activity bands were visible after soaking the gel in 1 M NaCl solution for 1 h.

Chromatography of the end products of xylan and CMC

Xylan (1%) was digested with the enzyme C108 (in Phosphate buffer pH 6.0) at 40 °C for 45 min of incubation. The end products were then analysed on silica gel 60 (GF_{254}) (Merck) thin-layer chromatography. After developing the products with Chloroform-Acetic acid-distilled water (6:7:1, v/v/v), spots were visualized by spraying aniline (1% v/v), diphenylamine (1% w/v), orthophosphoric acid (10% v/v), and a mixture prepared in acetone and baking the TLC plate in an oven at 160 °C for 30 min (20). To determine the cellulosic activity of the enzyme, the CMC (1%) was incubated with X13 endoxylanase for 45 min and the end product was analysed.

Results and discussion

The isolated strains producing extracellular xylanase activity on agar plate were identified as *Bacillus* sp. The *Bacillus* sp. X13 strain, showing the biggest xylanolytic potential, was gram-positive, rod shaped, motile, spore forming, and aerobic. The growth observed between pH 5.0 and 11.0 with an optimum at pH 7.0. Although the enzyme synthesis occurred at temperatures between 20 °C and 60 °C at pH 7.0, it was determined that the optimum temperature for growth and enzyme production was 40 °C.

Determination of the molecular weight

The extracellular xylanase of *Bacillus* sp. X13 was analysed by SDS-PAGE and zymography (Figure 1). Four different active xylanase bands were defined, which have relative estimated molecular masses as 68.5 kDa, 80.6 kDa, 95.5 kDa and 108.4 kDa (Figure 1). It has also been reported that more than 1 active xylanolytic enzyme bands with different molecular weight from *Bacillus* sps. (7,21,22). Although several reports regard endoxylanases as having a small molecular weight (23,24), in terms of xylanase classification studies, members of the 'family 10' typically have a high molecular weight (25).

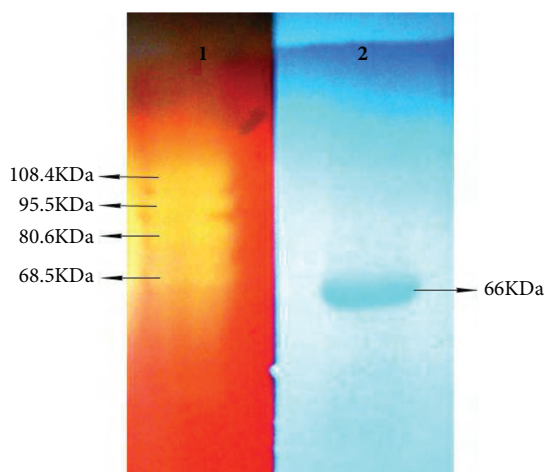


Figure 1. Zymogram analysis of X13 Endoxylanase. Xylanase was run on SDS-PAGE containing xylan, after electrophoresis the gel was cut into two pieces. Lane 1: X13 Endoxylanase enzyme activity zones. Lane 2: Bovine Serum Albumin used as marker.

Properties of the enzyme

The optimum pH was determined in four different buffer systems. The enzyme showed a significant relative activity of around 90% between pH 4.0 to 7.0 with an optimal level of 6.0. Although the average relative activity between pH 4.0 and 9.0 was 77%, over 90% of relative activity was observed between pH 4.0 and 6.0 (Figure 2). While the optimum temperature was observed around 40 °C (Figure 3), the enzyme

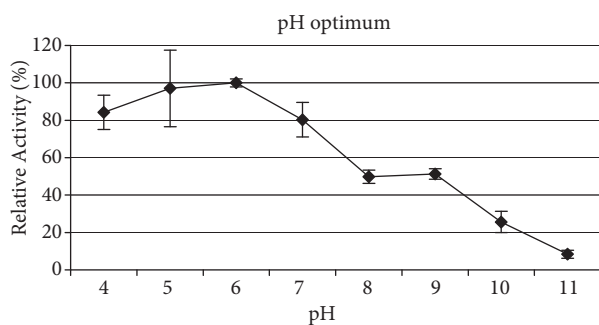


Figure 2. Effects of pH on the activity of *Bacillus* sp. X13 Endoxylanase. The reaction mixture contained 0.5 mL substrate (Oat spelt Xylan, 1%) and 0.5 mL enzyme. The reaction mixture was incubated at 40 °C for 30 min. The buffers were 100 mM Na-Acetate Acetic acid (pH 4.0-5.0), 100 mM Na-Phosphate buffer (pH 6.0-7.0), 100mM Tris-HCl buffer (pH 8.0-10.0), and 100 mM Borax-NaOH buffer (pH 11.0).

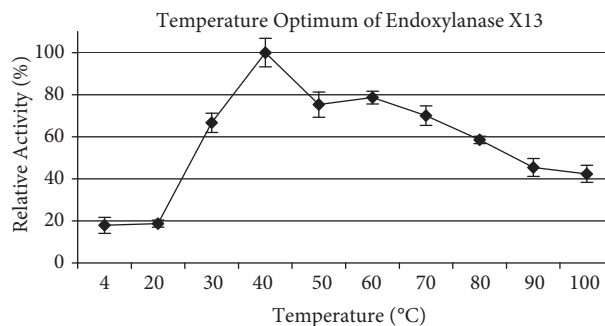


Figure 3. Effects of temperature on the activity of *Bacillus* sp. X13 Endoxylanase. The reaction mixture contained 0.5 mL substrate (oat spelt xylan, 1%, in Na-Phosphate buffer, pH 6.0) and 0.5 mL enzyme. The mixture was incubated for 30 min at temperatures from 4 to 100 °C under standard enzyme assay conditions.

has a broad temperature range between 30 and 80 °C by showing over 74% activity. Between the temperatures of 40 and 60 °C, the enzyme was highly active with an average of 85%.

For the thermal stability estimation, the enzyme was pre-incubated at temperatures between 20 and 100 °C for 15 and 60 min at the optimum pH value at which the remaining activity was determined (Figure 4). The enzyme was highly active up to 60 °C with a remaining average activity of 66% for 60 min. On the other hand, a gradual decrease was obtained with temperature increasing, more than 50% of the original activity was also lost over 50 °C after heat treatment for both periods. Although there are some reports

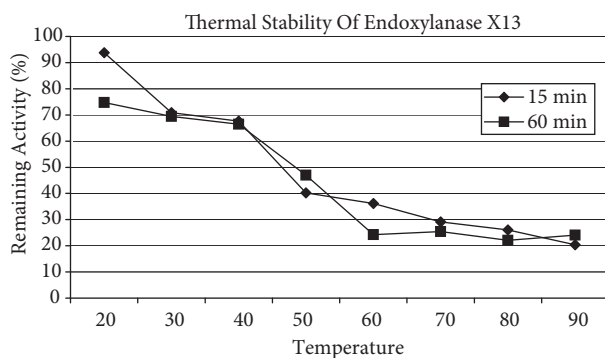


Figure 4. Thermal stability of *Bacillus* sp. X13 Endoxylanase. The enzyme was pre-incubated at temperatures from 20 to 90 °C for 15 (♦) and 60 min (■) at optimum pH, subsequently remaining activity (%) was determined under standard enzyme condition.

about xylanases with higher temperature stability (26, 27), X13 xylanase showed a rational thermal stability for most applications. The pH stability of the enzyme was determined by incubating it at 40 °C for 15 and 60 min, and 24 h. Remaining activity was measured by the standard assay methods. An average of 91% remaining activity was observed between pH 3.6 and 10.0 (Figure 5). Even though the average of 71% of the original activity was preserved for 60 min between pH 3.6 and 10.0, the maximal remaining activity was obtained at pH 5.0 and 6.0 for 15 min.

Effects of metal ions and chemicals on activity

The enzyme was incubated at 40 °C for 15 and 30 minutes at different concentrations of the metal ions and various chemicals prior to the standard enzyme assay. The activities measured were expressed as residual activity. The activity was fairly inhibited by the presence of SDS, which is similar to Xyl 1 (28), but highly inhibited with urea. The inhibition in the presence of urea points out that the enzyme consists of hydrophobic aminoacid composition (14). The additions of EDTA, CaCl₂ and Triton X-100, ZnCl₂, KCl, Na₂SO₃, PMSF, and 2-Mercaptoethanol did not significantly affect the enzyme activity, so that the residual activity was over 80% of the original activity (Figure 6).

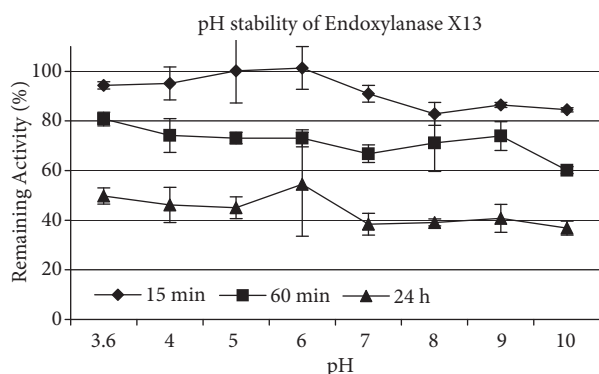


Figure 5. Effects of pH on the stability of *Bacillus* sp.X13 Endoxylanase. For determination of pH stability of endoxylanase X13, the enzyme was pre-incubated in buffers at 40 °C for 15 (♦), 60 min (■) and 24 h (▲). The buffers used were 100 mM Citrate-Phosphate (pH 3.6–5.0), 100 mM Na-Phosphate (pH 6.0–7.0), and 100 mM Glycine-NaOH (pH 8.0–10.0).

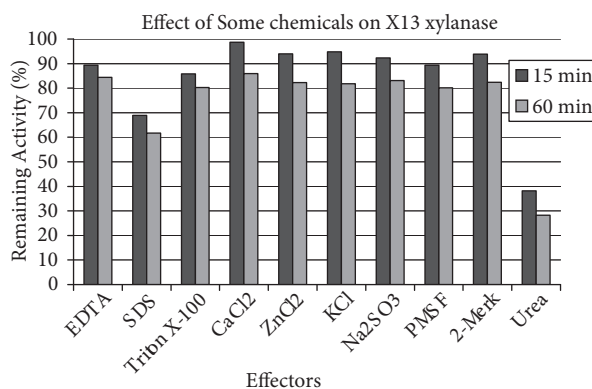


Figure 6. Effects of some metal ions and chemicals on the activity of *Bacillus* sp. X13 Endoxylanase.

Analysis of the end products of substrate hydrolysis

Hydrolysis products from oat spelt xylan were analysed by silica gel thin layer chromatography. After 45 min of incubation of the enzyme with the substrate mixture, xylose and other oligosaccharides were the main products detected (Figure 7a) that shows the enzyme is highly active for the production of xylose units from a xylose chain. This endoxylanase, like most endoxylanases from other *Bacillus* spp., released xylose from xylan, although some endoxylanases cleaves xylan into xylobiose or longer oligosaccharides. The density of xylose spot on TLC analysis (Figure 7a) could infer that the enzyme has more than one substrate binding sites, which is a typical property of family 10 (25).

When the enzyme, xylanase, incubated with CMC (1%, w/v), cellobiose was the shortest unit detected (Figure 7b), which indicates that the enzyme has multifunctional properties. Substrate specific studies have revealed that enzymes belonging to the family 10 may not be entirely exclusive to xylan and may also be active in cellulose substrates (29, 30).

Bacillus sp. used for the present study produced highly active multifunctional endoxylanase. It is very probable that the resulting endoxylanase activity of *Bacillus* sp. X13 was exclusively xylose producing, and has a favorable affect on CMC hydrolysis, as shown in this study, which may contain promising properties that could be used for many applications. Although there are some reports considering cellulase free

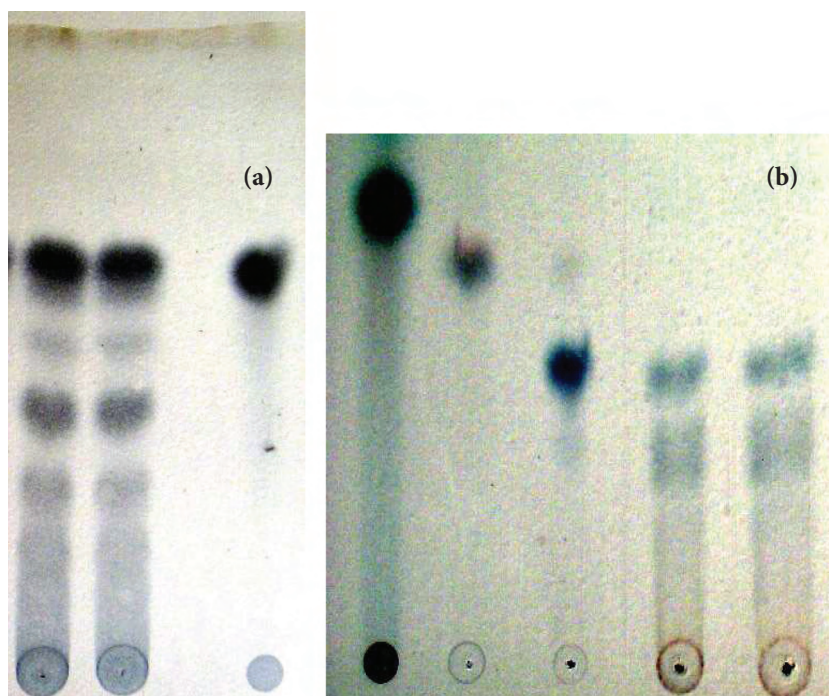


Figure 7. Thin layer chromatography of enzyme products from *Bacillus* sp. X13 Endoxylanase. (a) Hydrolytic activity of Endoxylanase X13 on xylan. Xylose: 5 mL, (1%, w/v); X13: 5 mL of X13 enzyme and substrate (Oat spelt xylan, 1%) mixture. (b) Hydrolytic activity of X13 on CMC. Xylose (1%, w/v), 5 mL; Glucose (G1), 5 mL; Maltose (G2), 5 mL; Enzyme (X13) and substrate (CMC, 1%) mixture, 5 mL.

xylanases (19), multifunctional enzymes are important in degradation of agricultural waste and digestibility of animal feed stock. It was concluded that the cellulose/xylan binding domain was a significant factor in the degradation process of insoluble cellulosic material. Enzymes of this type are classified in the family 10 of the glycosyl hydrolases, consisting of nonspecific glucanases that are able to hydrolyze xylan and numerous glucose-derived substrates, such as cellulose (31). According to White et al. (32), catalytic efficiency of these enzymes indicates that the action is mainly on xylan.

In conclusion, the results suggest that *Bacillus* sp. X13 was capable of producing endoxylanase showing cellulolytic activity. Thus, the crude xylanases extract from this bacterium may potentially be applicable in enzymatic hydrolysis of xylan especially in food industries, sugar production, and bioconversion of lignocellulosic materials. Moreover, we believe that further research in this area would be beneficial for

the improvement of animal feedstocks digestibility as well as the engineering of multifunctional enzyme studies.

Acknowledgements

This work was made possible by the support of Çukurova University Scientific Research Project Fund (FBE 2003-D-18). The authors would like to thank Dr. Emin Ozkose and Mr. Mustafa Tomak for some material they supplied.

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