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NOTES

Cloning and DNA Sequencing of *xyaA*, a Gene Encoding an Endo-β-1,4-Xylanase from an Alkalophilic *Bacillus* Strain (N137)

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The gene *xyaA* encoding an alkaline endo-β-1,4-xylanase from an alkalophilic *Bacillus* sp. strain (N137) isolated in our laboratory was cloned and expressed in *Escherichia coli*. The nucleotide sequence of a 1,656-bp DNA fragment containing *xyaA* was determined, revealing one open reading frame of 993 bp that encodes a xylanase (XyaA) of 39 kDa. This xylanase lacks a typical putative signal peptide, yet the protein is found in the *Bacillus* culture supernatant. In *Escherichia coli*, the active protein is located mainly in the periplasmic space. The xylanase activity of the cloned XyaA is an endo-acting enzyme that shows optimal activity at pH 8 and 40°C. This activity is stable at a pH between 6 and 11. Incubations of XyaA at 40°C for 1 h destroyed 45% of the activity.

Microbial degradation of xylans requires the collaboration of several kinds of enzymes such as β-1,4-xylanases, α-arabinofuranosides, β-xylosidases, α-glucuronidases, and esterases (11, 26). Among them, xylanases are the most studied because of their potential industrial applications.

In this paper, we report the cloning in *Escherichia coli* and sequencing of an endo-β-1,4-xylanase gene from an alkalophilic *Bacillus* sp. strain, N137. This gene is not expressed from its own promoter in *E. coli*, where the active protein XyaA is located mainly in the periplasmic space, although at least half of the produced protein is accumulated as an inactive form in inclusion bodies.

To determine the xylanase activity produced by *Bacillus* sp. strain N137 (25), this strain was grown in PY medium (3) supplemented with 0.5% oat spelt xylan, and samples were withdrawn periodically. The highest level of xylanase production, 1.2 U/mg, was obtained after 36 h of growth (Fig. 1A). Zymogram analysis was made on a renatured sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 0.2% soluble oat spelt xylan (20), and at least three bands of activity were detected (Fig. 1B). The sizes of these proteins were 64, 40, and 28 kDa.

A *Bacillus* sp. strain N137 library was made in *E. coli* MC 1061, and several clones producing different glycanases were selected by plating the transformants on Luria-Bertani (LB) plates supplemented with different substrates (25). One of the xylanase-producing clones was selected, and the plasmid isolated was subcloned and sequenced. One open reading frame of 993 nucleotides that encodes a protein of 331 amino acids was detected. The gene identified was denominated *xyaA* (Fig. 2). The predicted size of the protein (39 kDa) is in full agreement with the molecular mass (40 kDa) determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The 5′ end of the mRNA was identified as either nucleotide 366 or 367 (data not shown). The putative promoter regions −35 (TTG TGA) and −10 (CCAAGT) (Fig. 2) resemble σ^A *Bacillus* promoters (18).

The deduced protein was found to be similar to xylanases included in family 10 of glycosyl hydrolases (9, 10). The more similar proteins are those encoded by the following genes: *xynA* from *Caldocellum saccharolyticum* (45.9% similarity) (14), *xynA* from *Bacillus* sp. strain C-125 (40.2% similarity) (8), *xynA* from *Thermoanaerobacterium saccharolyticum* (37.5% similarity) (12a), *xynB* from *Butyrivibrio fibrisolvens* (29.6% similarity) (17), *xynZ* from *Clostridium thermocellum* (26.9% similarity) (5), *xlnA* from *Streptomyces lividans* (25.1% similarity) (22), and *xynA* from *Pseudomonas fluorescens* (22.4% similarity) (7) (Fig. 3). Of all of the enzymes compared, a high proportion of Asp and Glu residues were conserved. The predicted importance of these residues in catalysis has been tested by some authors. Lee et al. (12), working with a xylanase encoded by *xynA* from *T. saccharolyticum*, demonstrated that the substitution of D-537 and D-602 by N and E-600 by Q completely destroyed endoxylanase activity, suggesting that these three amino acids form a catalytic triad that operates in a general acid catalysis mechanism.

XyaA does not have the typical N-terminal signal peptide that occurs in most of the secreted proteins, suggesting the possibility that this protein could be intracellular. In this case, the XyaA specificity would be restricted to some xylo-oligosaccharides able to pass through the membrane into the cell. However, the use of thin-layer chromatography plates to analyze the degradation products obtained from oat spelt xylan revealed that even in long incubations, the smallest oligosaccharide obtained was xylotriose (data not shown). This result indicates a normal endoxylanase behavior. Supporting the secreted condition of this protein, polyclonal antibodies raised against a xylanase of *Streptomyces halstedii* JM8 (19) were able to detect the protein XyaA in *Bacillus* sp. strain N137 supernatant and not in cellular extracts (data not shown). All of these results suggest that XyaA is able to use a secretion system different from the ones described previously for *Bacillus*

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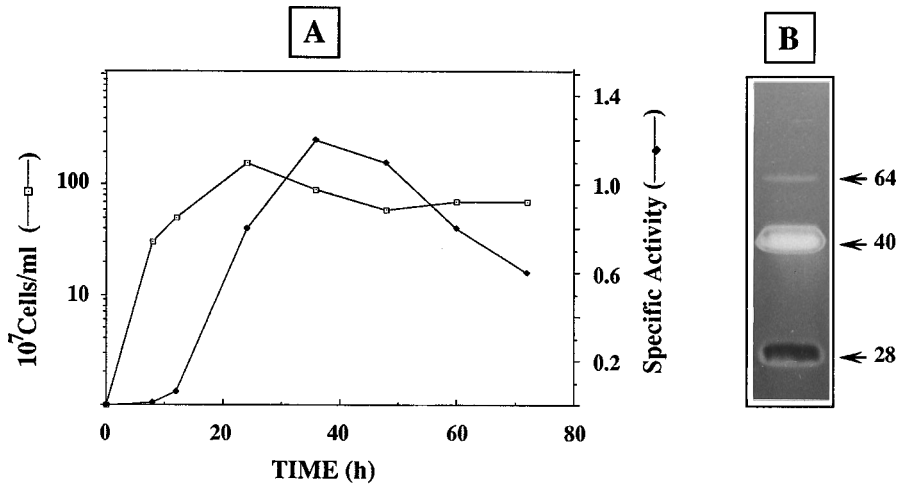

FIG. 1. (A) Growth of *Bacillus* sp. strain N137 in PY medium supplemented with 0.5% oat spelt xylan and production of xylanase determined by the dinitrosalicylic colorimetric assay (1); (B) xylanase activity, on renatured SDS-polyacrylamide gels, of the proteins present in *Bacillus* sp. strain N137 culture supernatant after 36 h of cultures. Molecular sizes (in kilodaltons) are indicated on the right.

spp. (revised in reference 23). The secretion system of XyaA could be similar to that described for some proteins such as alkaline protease and lipase from *Pseudomonas aeruginosa* (2, 6), proteases from *Erwinia chrysanthemi* (13), and α-hemolysin from *E. coli* (16), which are secreted without an N-terminal signal sequence. These proteins do contain a C-terminal targeting signal which is essential for secretion. These regions do not reveal any universally conserved primary structural ele-

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   1  ATCCTCGTAGTGGAACGGAGCGGAAGCCACTCGATTCTGCGGGATTTAGAGGAAGGCCACTGAAAAAGGTGTGTTTTTCACCTTTATCAGTGGTCTCTAA

 101  GCAATGAGCGAAACCGTTGCAATCTTTTTAAAGCCTGCTATGAGTGGGTTTCTCATAGCAGGCACGCAACGTGTGAAGCCATTGCCGCTTCCTTGAATCT

 201  GCTAAAAAGGACTTTTTCAGTGGCCTTATTTAGAGGTAGGGTCGAGACCCGACAGGCGCAGTGAGGAGGCTCGACACCTCCCCGCGGAAAGCGAGTGGCT

   1                        -35                -10                    R.B.S.          M  N  Q  Q  L
 301  GGAGCGTAATGGAACGAACATTTGGAGACGAACCTATAAAAACACCAAGTTTACGAAAACAGCCTTTAAAAAAGGAGGCAAACAAGCATGAATCAGCAAC

   6   N  I  P  N  L  Y  E  I  Y  K  D  F  F  S  I  G  A  A  V  N  S  K  T  L  E  S  E  K  E  L  L  K  K
 401  TTAACATTCCTAATCTTTATGAGATTTATAAAGACTTCTTTTCTATAGGTGCTGCAGTAAACTCTAAGACACTAGAATCAGAAAAAGAGTTATTAAAAAA

  39   H  Y  N  S  L  T  A  E  N  E  M  K  F  E  L  L  Q  P  E  Q  G  N  F  N  F  T  Q  A  D  K  L  V  A
 501  GCATTACAATAGCTTAACAGCAGAAAATGAAATGAAGTTTGAACTGTTACAGCCAGAACAAGGTAACTTTAACTTTACACAAGCCGACAAGCTTGTTGCA

  72   F  A  N  E  H  N  M  K  L  R  G  H  T  L  V  W  H  N  Q  T  T  G  W  L  F  Q  N  S  D  G  T  Q  V  N
 601  TTTGCAAACGAACACAATATGAAGCTACGAGGGCATACGCTTGTTTGGCACAACCAGACGACAGGATGGTTATTTCAAAATTCCGATGGAACACAAGTAA

 106   R  E  T  L  L  Q  R  M  E  A  H  I  S  T  V  L  G  R  Y  K  G  Q  F  Y  S  W  D  V  V  N  E  A  I
 701  ACCGTGAAACACTCTTACAGCGAATGGAAGCGCACATTTCAACCGTTTTAGGTCGTTATAAAGGCCAGTTCTATAGCTGGGACGTCGTTAACGAAGCCAT

 139   S  D  D  D  S  E  Y  L  R  K  S  K  W  L  D  I  I  G  E  D  F  I  A  K  A  F  E  F  A  H  Q  A  D
 801  TTCCGATGATGATTCTGAATACCTTCGTAAGTCTAAATGGTTAGATATTATTGGAGAGGATTTTATAGCAAAGGCTTTCGAATTTGCACATCAGGCTGAC

 172   P  N  A  S  L  F  Y  N  D  Y  N  E  S  H  P  N  K  R  E  R  I  Y  R  L  V  K  S  L  L  D  K  D  V  P
 901  CCTAATGCCTCTTTATTTTACAACGATTACAACGAATCTCATCCCAATAAACGAGAGAGAATCTATCGTCTCGTAAAATCTTTACTAGATAAAGACGTAC

 206   I  H  G  V  G  L  Q  A  H  W  N  V  H  D  P  S  L  D  D  I  R  A  A  I  E  R  Y  A  S  L  G  I  Q
1001  CTATTCACGGAGTAGGATTACAAGCTCACTGGAATGTTCACGATCCAAGTTTAGATGATATTCGAGCTGCAATAGAACGCTATGCTTCACTCGGGATACA

 239   L  Q  I  T  E  M  D  V  S  M  F  S  W  D  N  R  R  A  D  L  T  Q  P  T  E  K  M  L  N  L  Q  E  E
1101  GCTTCAAATAACAGAGATGGATGTTTCAATGTTTAGCTGGGATAATCGACGTGCAGACCTCACACAGCCTACAGAAAAAATGCTAAACCTACAAGAAGAA

 272   R  Y  D  Q  F  F  Q  L  F  R  E  Y  K  D  V  I  S  N  V  T  F  W  G  A  N  D  S  Y  T  W  L  N  D  F
1201  CGTTACGACCAATTCTTCCAATTATTTAGAGAATACAAGGATGTAATTTCTAATGTAACTTTCTGGGGTGCAAATGATTCTTATACGTGGTTAAATGATT

 306   P  V  R  G  R  K  N  W  P  F  I  F  D  E  K  G  D  P  Q  R  V  F  L  E  N  R  *
1301  TTCCTGTTAGAGGAAGAAAAAATTGGCCTTTCATTTTTGATGAAAAGGGTGACCCGCAAAGAGTCTTTTTGGAGAATCGCTAAATTTTAGGCTTCCTCTC

1401  CTCTCTTTAATAGTAAAACAATCCGGTTCGCTAAGCGAGCCGGGATTGTTTTGCTATCTCTTCGTTGTATCTTCTTTTAATCCTAAAATCCAAGTTGCAA

1501  TAAAAGCACCCACCGTAGCAATTAACATTCCAATTAGATAATGAATAATATTAGCCGTTCCAAATGGCGCTGCGATCGCGATCATTGGAATCCCTGTTAA

1601  ACCATAGGCGTTTACAATAACATTGATAAACACAACATATGCCCCACCTAGAGCTC
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FIG. 2. Nucleotide and deduced amino acid sequences of the *Bacillus* sp. strain N137 *xyaA* gene and its flanking regions. The −35 and −10 sequences are located upstream from the transcriptional start point, which was determined by primer extension (↓). The putative −35 and −10 regions and ribosome binding sites (R.B.S.) are shown in boldface type. Facing arrows, at the end of *xyaA*, mark an inverted repeat sequence that can work as a putative transcriptional terminator.

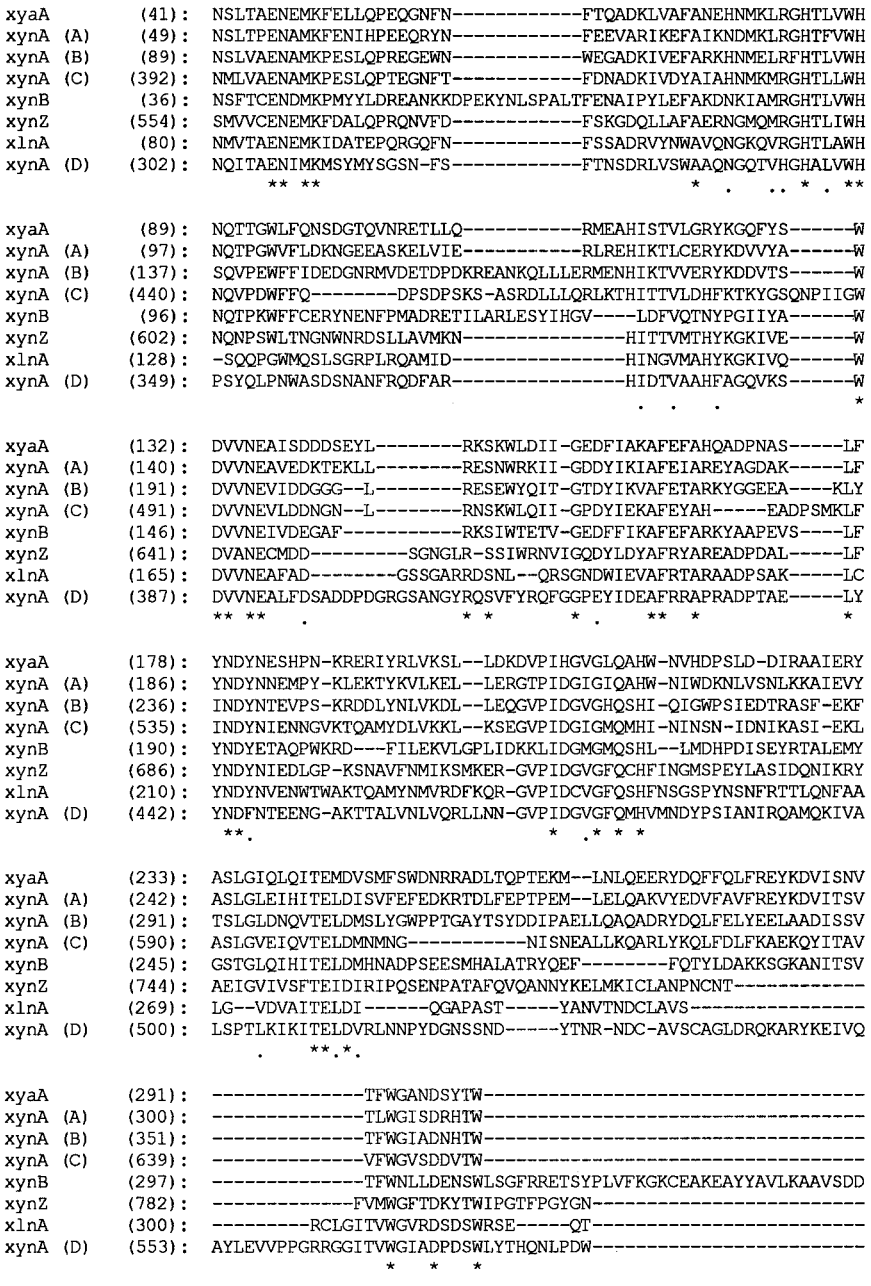

FIG. 3. Amino acid alignment, using the CLUSTAL program, of sequences belonging to several β-1,4-xylanases. Numbering of the amino acids starts in the N termini of all of the proteins. The introduction of gaps is indicated by dashes. Asterisks and dots below the alignment lines indicate identical and conserved amino acids residues, respectively, among all of these xylanases. Gene abbreviations: *xyaA*, *xyaA* from *Bacillus* sp. strain N137; *xynA* (A), *xynA* from *Caldocellum saccharolyticum* (14); *xynA* (B), *xynA* from *Bacillus* sp. strain C-125 (8); *xynA* (C), *xynA* from *T. saccharolyticum* (12a); *xynB*, *xynB* from *Butyrivibrio fibrisolvens* (17); *xynZ*, *xynZ* from *Clostridium thermocellum* (5); *xlnA*, *xlnA* from *S. lividans* (22); and *xynA* (D), *xynA* from *P. fluorescens* (7).

ments, but they all contain a putative amphipathic α-helix (4, 15, 24). XyaA has a small α-helix at its carboxy terminus, as do these other secreted proteins, although it does not have the domain GGXGXDXXX (where X is any amino acid) that is repeated several times in the C termini of all of the others. The possibility of an arrangement of the DNA that encodes the N terminus of XyaA during the cloning experiments was discarded by Southern analysis in which the cloned fragment and *Bacillus* sp. strain N137 genomic DNA showed identical restriction fragments (data not shown).

E. coli carrying a plasmid that contains the xylanase open reading frame was grown in LB medium for 16 h and fractionated as described by Sambrook et al. (21). Enzymatic activity was measured in the cytoplasmic and periplasmic fractions and in the extracellular supernatant. The xylanase activity was found mainly in the periplasmic fraction, indicating that the

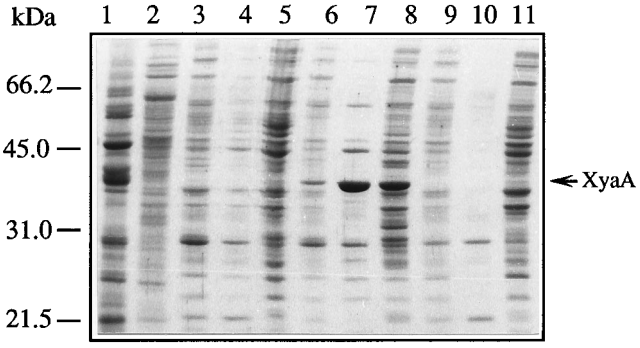

FIG. 4. SDS-PAGE gel stained by Coomassie brilliant blue R-250 of *Bacillus* sp. strain N137 supernatant (lane 1) and cell extract (lane 2) and *E. coli* DH1 fractions, namely, supernatant (lanes 3, 6, and 9), periplasmic (lanes 4, 7, and 10), and cytoplasmic (lanes 5, 8, and 11). Plasmids used included *E. coli*/pBluescript KS^+ (lanes 3 to 5), *E. coli* DH1/pCT121 (lanes 6 to 8; the gene *xyaA* is in the same orientation as that of the *lac* promoter), and *E. coli* DH1/pCT123 (lanes 9 to 11; the gene *xyaA* is in the opposite orientation to that of the *lac* promoter). The arrow indicates the position of the protein XyaA.

secretion signals of this protein must be recognized by the secretion system of *E. coli* (Fig. 4). However, SDS-PAGE of the proteins present in these fractions showed that XyaA is also present in the cytoplasmic fraction in an inactive form. Further analysis demonstrated that the protein present in the cytoplasmic fraction is accumulated in inclusion bodies (data not shown). The use of plasmids carrying the DNA insert in opposite orientation to that of the *lac* promoter permitted us to corroborate that this gene is expressed only when its orientation is coincident with the *lacZ* promoter (Fig. 4, lanes 9, 10, and 11). This result suggests that the XyaA promoter is not functional in *E. coli.*

The enzymatic properties of XyaA at different pHs and temperatures were examined on the periplasmic fraction of *E. coli* carrying this gene. The crude enzyme is active (at least 90%) over a wide pH range (from pH 6 to 10). It has optimal activity at pH 8, and it retains more than 85% of the activity even at pH 11 (Fig. 5A). The stability at several pHs was studied by incubation of the same *E. coli* fraction at different pHs without substrate for 1 h at 24°C, and the residual activity was measured at pH 9. This enzyme is stable between pH 6 and 11 for at least 1 h (Fig. 5A). The activity of the crude enzyme was also measured at different temperatures. The optimum reaction temperature of this enzyme was 40°C (Fig. 5B). However, thermostability assays demonstrated that this enzyme lost 45% of its activity after incubation for 1 h at 40°C and became completely inactive if the incubation took place at a temperature over 50°C (Fig. 5B).

Nucleotide sequence accession number. The DNA sequence shown in Fig. 2 has been submitted to the EMBL database under accession number Z35497.

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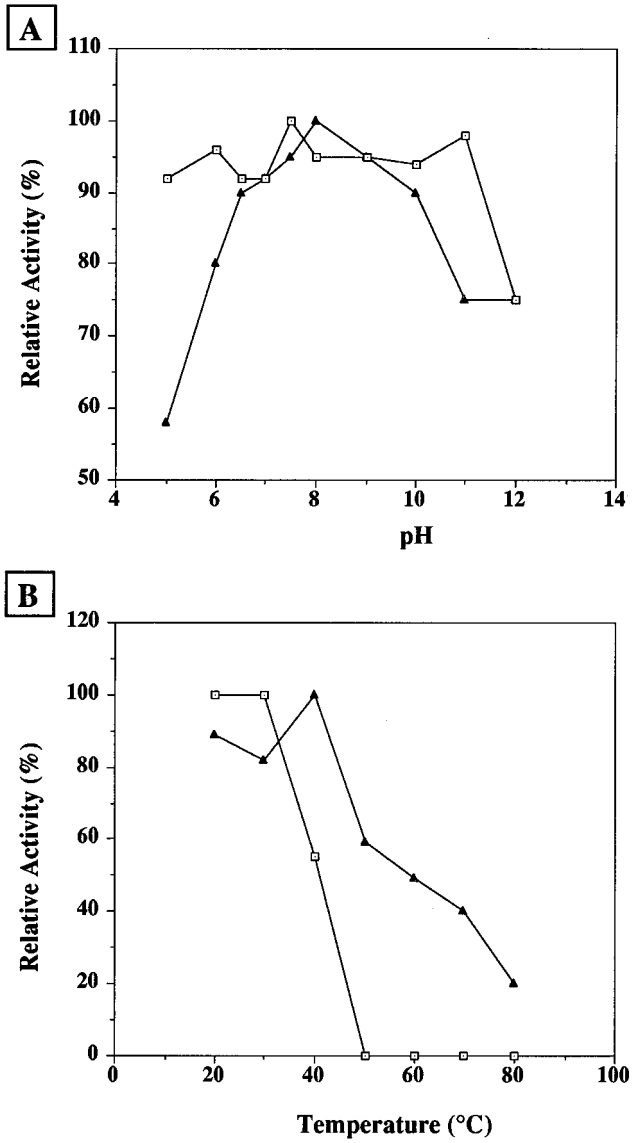

FIG. 5. Effect of pH (A) and temperature (B) on activity (▲) and stability (⊡) of XyaA. The buffers used were sodium acetate at pH 5, phosphate buffer from pH 5.5 to 8, glycine-NaOH from pH 8 to 10, and glycine-NaOH-NaCl from pH 10 to 12. The final concentrations of all buffers were 200 mM. The stability at several pHs was determined after preincubation for 1 h at different pHs and at room temperature without the substrate, and the activity was later assayed at 40°C and pH 9. The stability at different temperatures was determined at pH 9 for 1 h, and the activity was later assayed at 40°C.

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