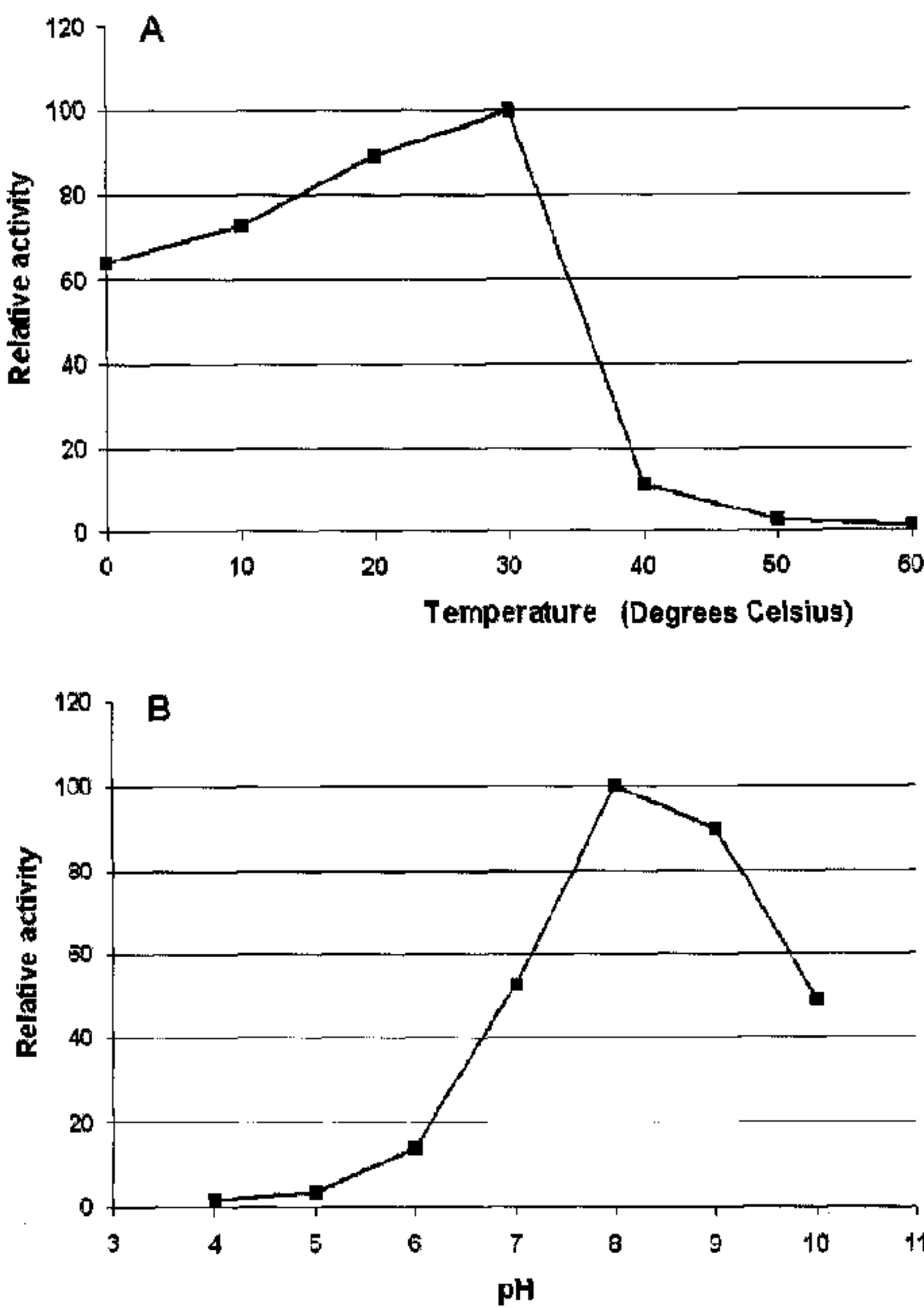




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(54) Titre : BETA-GALACTOSIDASE ACTIVE A FROID, SON PROCEDE DE FABRICATION ET UTILISATION D'UNE TELLE ENZYME
(54) Title: COLD-ACTIVE BETA-GALACTOSIDASE, A METHOD OF PRODUCING SAME AND USE OF SUCH ENZYME



(57) **Abrégé/Abstract:**
There is provided a novel cold-active beta-galactosidase enzyme specific for lactose. The enzyme is thus useful in e.g. the food industry for catalyzing at low temperatures the hydrolysis of lactose disaccharide into its constituent monosaccharides, glucose and galactose. The present invention further provides a method of producing the cold-active beta-galactosidase by recombinant DNA technology.

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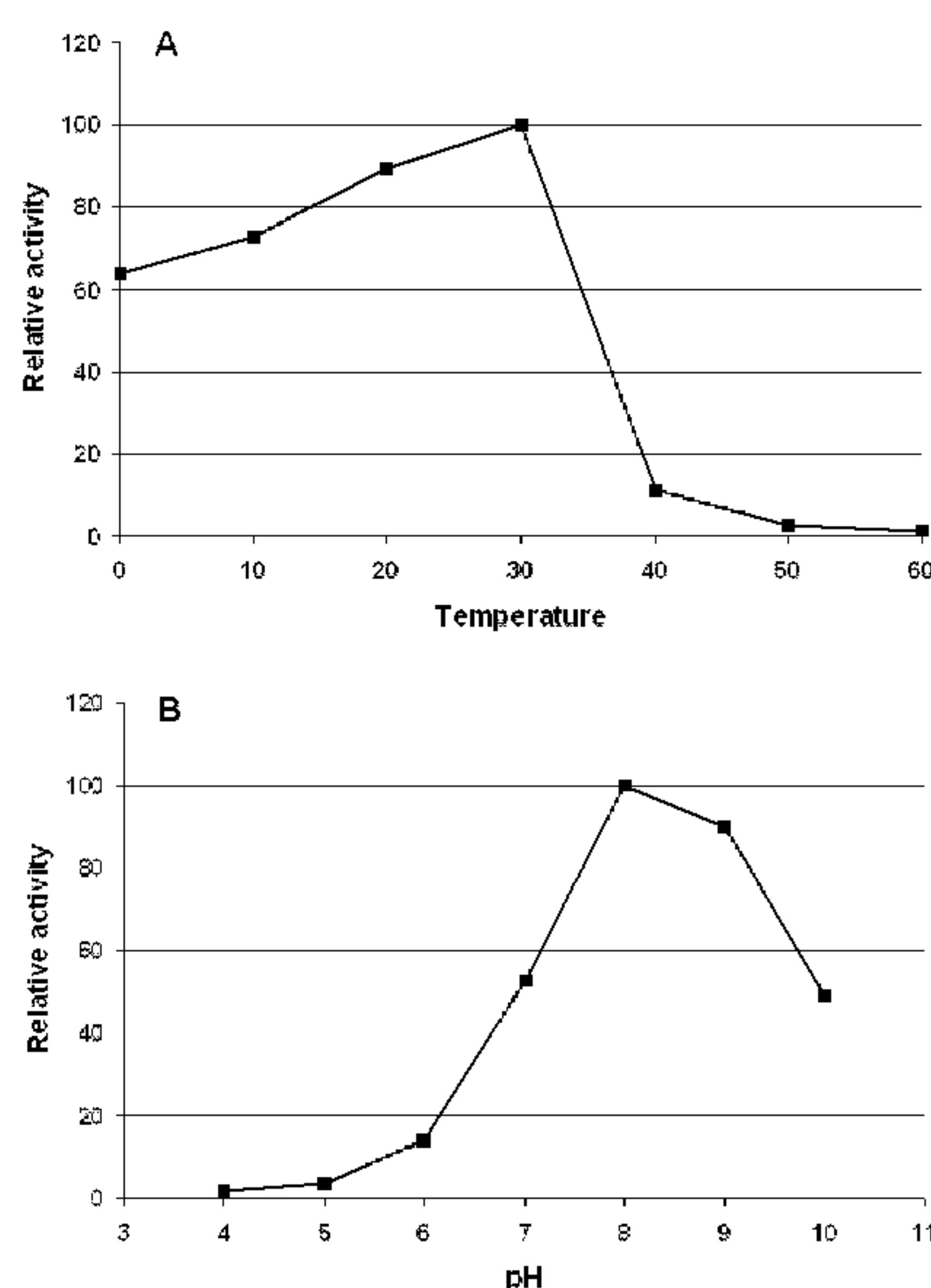
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Figure 14



(57) Abstract: There is provided a novel cold-active beta-galactosidase enzyme specific for lactose. The enzyme is thus useful in e.g. the food industry for catalyzing at low temperatures the hydrolysis of lactose disaccharide into its constituent monosaccharides, glucose and galactose. The present invention further provides a method of producing the cold-active beta-galactosidase by recombinant DNA technology.

COLD-ACTIVE BETA-GALACTOSIDASE, A METHOD OF PRODUCING SAME AND USE OF SUCH ENZYME

5 FIELD OF THE INVENTION

The present invention relates to a novel cold-active beta-galactosidase enzyme specific for lactose. The enzyme is thus useful in e.g. the food industry for catalyzing at low
10 temperatures the hydrolysis of lactose disaccharide into its constituent monosaccharides, glucose and galactose. The present invention further provides a method of producing the cold-active beta-galactosidase by recombinant DNA technology.

15

BACKGROUND OF THE INVENTION

Beta-galactosidase (beta-D-galactoside galactohydrolase, EC
20 3.2.1.23) is an enzyme capable of hydrolyzing the disaccharide lactose to its monosaccharide constituents, D-glucose and D-galactose. Beta-galactosidases are found in a large variety of organisms, like mammals, plants, fungi, yeasts, and bacteria. In Nature, beta-galactosidases
25 hydrolyze lactose and other D-galactose-containing carbohydrates. In the industry, beta-galactosidases have been used primarily within the food industry. Beta-galactosidase hydrolysis of lactose and lactose-containing dairy products are used throughout in the dairy industry in
30 the preparation of lactose-free or low-lactose products, which may be consumed by humans suffering from lactose intolerance. Hydrolysis of lactose by beta-galactosidases may also be used in applications where the removal of

lactose is required, i.e. prevention of crystallisation of lactose in food and removal of D-galactose moieties in glycosylated proteins. Other applications of beta-galactosidases comprise hydrolysis of lactose into D-
5 galactose and D-glucose with the subsequent modification of the monosaccharides to high value products, like the sweetener D-tagatose (Jørgensen et al. 2004).

Application of beta-galactosidases could be used to produce
10 lactose-free and low-lactose dairy products for lactose intolerant humans.

The major applications for lactose hydrolysis are listed below.

15 a) Liquid milk. Lactose hydrolysis in liquid milk improves digestibility for lactose intolerant consumers. In flavoured milks, lactose hydrolysis increases sweetness and enhances flavours.

b) Milk powders. Lactose hydrolysed milk powders for
20 dietetic uses, especially for infants with temporary beta-galactosidase deficiency.

c) Fermented milk products. In some cases, lactose hydrolysis in milk used for the manufacture of cheese and yoghurt can increase the rate of acid development and thus
25 reduce processing time.

d) Concentrated milk products. Lactose hydrolysis in concentrated milk products (e.g. sweetened condensed milk, ice cream) prevents crystallisation of lactose.

e) Whey for animal feed. Lactose hydrolysis in whey
30 enables more whey solids to be fed to pigs and cattle and also prevents crystallisation in whey concentrate.

f) Whey. Lactose hydrolysed whey is concentrated to produce a syrup containing 70-75 per cent solids. This

syrup provides a source of functional whey protein and sweet carbohydrate and is used as a food ingredient in ice cream, bakery and confectionery products.

- 5 The conventional approach in food processing is to carry out the hydrolysis of lactose at 40 °C during approximately four hours.

However, milk or lactose solution as a raw material is a
10 preferable nutrition source for bacteria. As the result, the putrefaction owing to the saprophyte contamination during the treatment is a serious problem in the food production. Thus, the fact is that the conventional beta-galactosidase is of limited use.

15

Most beta-galactosidases in practical use are active only at temperatures above 20-30 °C, temperatures where food spoiling bacteria thrive at best.

- 20 Attempts to use thermophilic beta-galactosidases have been used but the products have suffered from off flavours and reduced organoleptic properties due to the heat treatment, and the processes have demanded high energetic costs.

- 25 A number of cold-active beta-galactosidases have been described from *Arthrobacter* (Coker, et al. 2003; Karasová-Lipovová, et al. 2003; Nakagawa et al. 2003; Nakagawa et al. 2006), from *Carnobacterium piscicola* (Coombs and Brenchley, 1999) and from *Pseudoalteromonas* (Cieslinski, et
30 al. 2005; Fernandes, et al. 2002; Hoyoux, et al. 2001; Turkiewicz, et al. 2003). Furthermore, Nakagawa et al. (2006) described a cold-active beta-galactosidase from the yeast *Guehomyces pullulans*. However, the activity of cold-

active beta-galactosidases described so far is low at the low temperatures, which is wanted by the dairy industry. The beta-galactosidase from the yeast *Guehomyces pullulans* had approximately 17% at 0 °C (Nakagawa et al. 2005), the
5 beta-galactosidases from *Carnobacterium piscicola* BA showed approximately 24% activity at 10 °C (Coombs and Brenchley, 1999) and the enzymes from *Pseudoalteromonas* isolates showed 39% activity (Fernandes et al. 2002), 22% activity (Cieslinski et al. 2005), and 12% activity (Hoyoux et al.
10 2001) at 10 °C. So far, the beta-galactosidases with highest activity at low temperatures have been isolated from Antarctic *Arthrobacter* isolates. Karasová-Lipovová, et al. (2003) showed that a psychrotolerant *Arthrobacter* sp. C2-2 isolate produced beta-galactosidase, which displayed
15 19% of its maximal activity at 10 °C, Coker et al. (2003) described an enzyme from an Antarctic *Arthrobacter* isolate with approximately 50% at 0 °C, and Nakagawa et al. (2003, 2006) described a beta-galactosidase from *A. psychrolactophilus* F2, which had its temperature optimum at
20 10 °C.

However, the cold-active beta-galactosidase from the Antarctic *Arthrobacter* was produced in low amounts in native cells and attempts to produce the enzyme
25 recombinantly in *E. coli* were unsuccessful since about 90% of the enzyme was located in insoluble inclusion bodies (Coker et al. 2003). The cold-active beta-galactosidase from *A. psychrolactophilus* F2 could be produced heterologously, but had lower activity than the other
30 *Arthrobacter* beta-galactosidases (Nakagawa et al. 2006).

Therefore, in order to develop a low-temperature process for hydrolysis of lactose there is a need for a novel cold-

active beta-galactosidase and a method for producing such enzyme.

5 SUMMARY OF THE INVENTION

The present invention has, by using recombinant DNA technology, for the first time made it possible to provide a cold-active beta-galactosidase with high specific
10 activity in industrially appropriate quantities for the manufacturing of food products and pharmaceuticals.

Accordingly, the present invention provides a purified cold-active beta-galactosidase, specific for lactose,
15 having a stable enzymatic activity at temperatures less than 8°C, and specifically at 4°C, which corresponds to refrigerating conservation temperature for dairy products. The enzyme of the present invention is consequently able to hydrolyse lactose in dairy products and milk processing at
20 such a low temperature that saprophytes are hindered to proliferate. The hydrolysis of lactose can be carried out in these refrigeration conditions with no need of a particular treatment to the dairy product concerned.

25 Specifically, the present invention provides a cold active beta-galactosidase having the sequence as defined in SEQ ID NO. 1, or one having at least 80% homology to the amino acid sequence as defined in SEQ ID NO. 1, the amino acid sequence being selected so that the enzyme has a stable
30 enzymatic activity at temperatures less than 8 °C.

Preferably the amino acid sequence has at least 90%, and more preferably 95%, homology to the amino acid sequence as defined in SEQ ID NO. 1.

In order to obtain the cold-active beta-galactosidase of the present invention there is further provided a DNA sequence, which

- 5 a) encodes a protein with an amino acid sequence as given in SEQ ID NO. 1, or
- b) hybridises under stringent or very stringent conditions to the sequence of a), or
- c) is degenerative of the sequence of a) or b)

10

Preferably the DNA sequence is derived from the genus *Alkalilactibacillus*, such as the species *Alkalilactibacillus ikkense*, and has a nucleotide sequence as given in SEQ ID NO. 2.

15

In a further embodiment, the present invention provides a recombinant vector comprising a DNA sequence that encodes a protein with an amino acid sequence as given in SEQ ID NO. 1, or one having at least 80% homology to the amino acid

20 sequence as defined in SEQ ID NO. 1, the amino acid sequence being selected so that the enzyme has a stable enzymatic activity at temperatures less than 8 °C.

Preferably the amino acid sequence has at least 90%, and more preferably 95%, homology to the amino acid sequence as

25 defined in SEQ ID NO. 1.

30

Another object of the present invention is a strain of an isolated *Alkalilactibacillus* bacterium capable of producing a cold-active beta-galactosidase according to the present

 invention. A preferable strain is *Alkalilactibacillus ikkense* deposited on the 3rd of March, 2009, under the Budapest Treaty at the BCCM/LMG-Collection (Belgian Coordinated Collections of Microorganisms) with the

Accession No LMG P-24866 and variants and mutants derived therefrom.

To purify the cold-active beta-galactosidase according to
5 the present invention, a bacterium living in the Greenland
area was isolated and characterised in order to study how
its enzymes, and particularly, the beta-galactosidase was
adapted to cold. These studies led to the purification of
the beta-galactosidase, meaning that this protein was
10 obtained substantially free of other proteins using protein
purification steps known in the art.

Thus, another object of the present invention is a strain
of an isolated Alkalilactibacillus bacterium capable of
15 producing a cold-active beta-galactosidase according to the
present invention. A preferable strain is
alkalilactibacillus ikkense.

Another object of the invention is a recombinant plasmid or
20 vector suited for transformation of a host, capable of
directing the expression of a DNA sequence according to the
invention in such a manner that the host expresses the
cold-active beta-galactosidase of the present invention in
recoverable form.

25

According to the invention, another object is the so
transformed host. A variety of host-expression systems may
be conceived to express the cold-active beta-galactosidase
coding sequence, for example bacteria, yeast, insect cells,
30 plant cells, mammalian cells, etc. Particularly, in yeast
and in bacteria, a number of vectors containing
constitutive or inducible promoters may be used.

It is also an object of the present invention to provide a process for purifying the cold-active beta-galactosidase according to the present invention from a bacterium as well
5 as to provide a process for producing cold-active beta-galactosidase according to the invention in a transformed host.

Accordingly, the invention pertains to a method of
10 producing a polypeptide having cold-active beta-galactosidase activity, comprising isolating a DNA fragment encoding the polypeptide, inserting said DNA fragment into an appropriate host organism, cultivating the host organism under conditions, which lead to expression of the a
15 polypeptide with cold-active beta-galactosidase activity and recovering said polypeptide from the cultivation medium or the host organism.

An appropriate host organism is preferably selected from
20 the group consisting of *Escherichia*, *Bacillus*, *Bifidobacterium*, *Lactococcus*, *Lactobacillus*, *Streptomyces*, *Leuconostoc*, *Streptomyces*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torula*, *Torulopsis* and *Aspergillus*.

25 In a further aspect, the invention relates to a recombinant DNA molecule comprising a DNA fragment encoding a polypeptide having cold-active beta-galactosidase activity and to a microbial cell comprising such recombinant DNA molecule.

30

In another aspect, the invention pertains to the use of the above polypeptide with cold-active beta-galactosidase activity or a microbial cell expressing such polypeptide in

the manufacturing of a food product or a pharmaceutical product.

In another useful aspect, there is provided a method for
5 reducing the lactose content of a food product, comprising
adding to the food product an amount of the polypeptide or
the microbial cell as disclosed herein, which is sufficient
to remove at least part of the lactose present in said food
product.

10

In a practical aspect, the invention pertains to the
inactivation of the beta-galactosidase activity of the
polypeptide by a moderate increase of the temperature.

15 In a further interesting aspect, there is provided a method
using the polypeptide harbouring a cold-active beta-
galactosidase activity or a microbial cell according to the
invention in the hydrolysis of lactose, whereby the
polypeptide and/or the microbial cell is applied to a
20 reactor containing lactose, which is hydrolyzed under low-
temperature conditions.

These and other objects of the present invention will be
apparent from the following disclosure.

25

Other characteristics of the present invention are listed
in the annexed claims.

30 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 discloses a phylogenetic tree of 16S rRNA gene
sequences from *Alkalilactibacillus ikkense* strain 517 and

its closest relatives within the rRNA group 1 in the phyletic assemblage classically defined as the genus *Bacillus*. Bootstrap (n=100) values are shown. Bar, 0.015 substitutions per nucleotide position.

5

Figure 2 shows temperature dependence of native Ikka-beta-galactosidase. Y-axis is the relative activity in percent of the maximal activity. X-axis is the incubation temperature.

10

Figure 3 shows temperature stability of native Ikka-beta-galactosidase. Y-axis is the residual activity left after incubation at the time indicated on the X-axis. ♦, ●, ▲, ■, ◇, ○, indicate incubation temperatures at 0, 10, 20, 30, 15 40, and 50 °C, respectively.

20

Figure 4 discloses pH dependence of native Ikka-beta-galactosidase. Y-axis is the relative activity in percent of the maximal activity. X-axis is pH in the beta-galactosidase assay.

25

Figure 5 shows SDS-PAGE of extracts from native *A. ikkense* cells induced with 1 mM IPTG (lanes 2 and 3) and from uninduced, native *A. ikkense* (lanes 4 and 5). Arrows in lanes 2 and 3 indicate the 120 kDa beta-galactosidase band. Lane 1 is molecular weight marker. The marker at 120 kDa is beta-galactosidase from *E. coli*.

30

Figure 6 shows SDS-PAGE of three extract dilutions from recombinant *E. coli* cells expressing Ikka-beta-galactosidase (lanes 4, 5, and 6). For comparison, enzyme extract dilutions with beta-galactosidase from *Kluyveromyces lactis* (lanes 7, 8, and 9) and a molecular

weight marker with native *E. coli* beta-galactosidase (marker at 120 kDa) were co-electrophoresed with the recombinant Ikka-beta-galactosidase. Arrows show the position of the 120 kDa beta-galactosidase bands

5

Figure 7 discloses temperature dependence of recombinant Ikka-beta-galactosidase produced in *E. coli*. Y-axis is the relative activity in percent of the maximal activity. X-axis is the incubation temperature. ♦, indicate recombinant Ikka-beta-galactosidase; o, indicate *Kluyveromyces lactis* beta-galactosidase.

10

Figure 8 shows specific activity of recombinant Ikka-beta-galactosidase produced in *E. coli*. Y-axis is specific activity in nmoles ONP released/hour/mg enzyme. X-axis is temperature in °C. ♦ is recombinant Ikka-beta-galactosidase, o is *Kluyveromyces lactis* beta-galactosidase.

15

Figure 9 discloses thermal stability of recombinant Ikka-beta-galactosidase enzyme. An equal amount of enzyme was incubated at the temperature indicated and samples were withdrawn at different time intervals. Y-axis is absorbancy at wavelength 415 nm. X-axis is time in minutes.

20

Figure 10 also discloses thermal stability of recombinant Ikka-beta-galactosidase enzyme, while the X-axis is time in hours.

25

Figure 11 shows pH dependence of recombinant Ikka-beta-galactosidase. Y-axis is absorbancy measured at wavelength 415. X-axis is pH in the beta-galactosidase assay.

30

Figure 12 shows hydrolysis of lactose by recombinant Ikka-beta-galactosidase produced in *E.coli*. The reaction mixture contained lactose in three concentrations, 1.25 mg/ml, 2.5 mg/ml and 5 mg/ml. Samples were withdrawn after incubation
5 for 15, 150, and 1440 minutes. The reactions were incubated at 5 °C (**A**) or 20 °C (**B**) and analysed by thin layer chromatography (TLC). The TCL plates were sprayed with an orcinol reagent, and hydrolysis of lactose was estimated by
10 the concomitant appearance of glucose and galactose spots.

Figure 13 shows SDS polyacrylamide gel electrophoresis of crude extract of *E. coli* cells expressing *A. ikkense* beta-galactosidase, Lane 1. The beta-galactosidase in the crude
15 extract was further purified on ion exchange chromatography, Lane 2, or on affinity chromatography, Lane 3. The arrow indicates the position of the *E. coli* 120 kDa beta-galactosidase band. Numbers to the left indicate the position of protein bands in the PageRuler™ Plus Prestained
20 Protein Ladder (Fermentas).

Figure 14 shows temperature dependence (A) and pH dependence (B) of the purified, recombinant *A. ikkense* beta-galactosidase and of a crude extract containing
25 recombinant enzyme. Y-axis is the relative activity in percent of maximal activity. X-axis is the incubation temperature (A) or pH (B). The relative activity of the purified, recombinant enzyme with ONPG as substrate is illustrated by black squares. Assays were performed in
30 triplicates, and standard error was below 0.05.

Figure 15 shows thermal stability of the purified, recombinant *A. ikkense* beta-galactosidase enzyme. An equal amount of enzyme was incubated at the temperatures indicated and samples were withdrawn at different time intervals. Y-axis is the residual activity in percent of maximal activity. Enzyme samples were incubated at temperatures from 0 °C to 60 °C and at the time points indicated (X-axis), samples were withdrawn and assayed for active beta-galactosidase at 20 °C. Assays were performed in triplicates, and standard error was below 0.05.

Figure 16 shows benchmarking the *A. ikkense* beta-galactosidase (black squares) with commercially available Lactozyme® 3 3000L from *K. lactis* (open circles). An equal amount of enzyme (2 mg/ml) was incubated with ONPG as substrate at temperatures from 0 °C to 20 °C. Samples were withdrawn at different time intervals and hydrolyzed ONP was measured at A420 nm. Hydrolysis efficiency was calculated as increase in A420 nm per min per microgram active enzyme. Assays were performed in triplicates, and standard error was below 0.05.

Figure 17 shows thin-layer chromatography (TLC) of the hydrolysis of lactose by the *A. ikkense* beta-galactosidase. Lanes 4-6: samples incubated at 5 °C for 2½ h (4: 1.25 mg/ml lactose, 5: 2.5 mg/ml lactose, 6: 5 mg/ml lactose). Lanes 7-9: samples incubated at 20 °C for 2½ h (7: 1.25 mg/ml lactose, 8: 2.5 mg/ml lactose, 9: 5 mg/ml lactose). Lanes 1-3: Controls, 0.0125 µg of each of the carbohydrates 13 lactose (lane 1), galactose (lane 2) and glucose (lane 3).

DETAILED DESCRIPTION OF THE INVENTION

"Beta-galactosidase" (beta-D-galactoside galactohydrolase,
5 EC 3.2.1.23) is defined as an enzyme capable of hydrolysing
lactose to the monosaccharides D-glucose and D-galactose.

"Cold-active" is defined as having activity at temperatures
at 15 °C and below, preferably at 10 °C and below and most
10 preferably at 5 °C and below.

A "host cell" is selected from a group of microorganisms
comprising fungi, yeasts, and prokaryotes. The
microorganism is more preferably a prokaryote and most
15 preferably a bacterium.

Conditions of incubating beta-galactosidase with lactose
are defined by performing incubation at a temperature
between 0 °C and 20 °C, preferably between 5 °C and 15 °C.
20

The term "stringent condition" refers to a condition such
that a hybridization is conducted in a solution containing
6xSSC (20xSSC represents 333 mM Sodium citrate, 333 mM
NaCl), 0.5% SDS and 50% formamide at 42 °C, and then the
25 hybridized products are washed in a solution of 0.1xSSC,
0.5% SDS at 68°C, or to conditions as described in
Nakayama, et al., Bio-Jikken-Illustrated, vol. 2, "Idenshi-
Kaiseki-No-Kiso (A Basis for Gene Analysis)", pp. 148-151,
Shujunsha, 1995.

30

EXAMPLES

EXAMPLE 1

5

Isolation of bacteria producing cold-active beta-galactosidases1.1 Sampling of bacteria

10

Ikaite material was collected by scuba divers in the Ikka Fjord, South West Greenland (61°11'N, 48°01'W) from a depth of approximately six to ten meters. The columns were between 36-70 cm in length and between 5 and 30 cm in diameter. The columns were kept cold during transportation to the field laboratory.

15

1.2 Screening bacteria for beta-galactosidase production

20

Approximately 3 cm³ of ikaite material from a slice 15-18 cm from the top of an ikaite column was drilled out and suspended in 250 ml R2 broth (Schmidt et al. 2006) buffered to pH 10 with 0.2 M Na₂CO₃/NaHCO₃ buffers as described by Stoll and Blanchard (1990). After incubation at 5 °C for 2 months the culture was inoculated onto R2 medium, pH 10 without glucose but supplemented with lactose (1% w/v), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 µg/ml), 10 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and agar (1.5 %, w/v). The plates were incubated at 5 °C for one to two weeks. A total of 17 blue colonies indicating production of beta-galactosidase were detected. Since 16S rRNA gene analyses of the seventeen isolates

25

30

showed identical sequences only one of the isolates, strain 517, was chosen for further characterization.

5 EXAMPLE 2

Taxonomical analysis of Isolate 517 and description of a new genus and species, *Alkalilactibacillus ikkense*

10 2.1 Phylogenetic analysis of 16S rRNA gene sequences

DNA for phylogenetic analysis was extracted from cells of isolate 517 using FastDNA SPIN Kit for Soil as described by the manufacturer (BIO 101, Irvine, CA). 16S rRNA gene
15 amplification was carried out using the primers 27F and 1492R (Lane 1991), and DNA sequencing was carried out at MWG Biotech AG (Ebersberg, Germany) using the same two primers plus additional primers 519R, 532F, 907F, and 907R (Lane 1991). The near full length DNA sequence of the 16S rRNA
20 gene from isolate 517 was submitted to GenBank/EMBL/DDBJ with the accession number EU281853. Related sequences were retrieved from public databases using BLASTn at the NCBI server. The closest
related 16S rRNA gene sequences were aligned using the
25 Clustal W multiple alignment program MegAlign 5.03 (DNASTAR, Inc., Madison, WI). The Clustal W analysis showed that the closest relatives were *Natronobacillus azotifigens* (accession no. EU850815) (Sorokin et al. 2008),
Paraliobacillus ryukyuensis (accession no. AB087828)
30 (Ishikawa et al. 2002), *Halolactibacillus halophilus* (accession no. AB196783) (Ishikawa et al. 2005),
Halolactibacillus miurensis (accession no. AB196784) (Ishikawa et al. 2005), *Amphibacillus tropicus* (accession

no. AF418602) (Zhilina et al. 2001, 2002), and
Gracilibacillus halotolerans (accession no. AF036922) (Wainø
et al. 1999). Isolate 517 was most closely related to *N.*
azotifigens, *P. ryukyuensis* and *A. tropicus* with 95,9%,
5 94.4% and 93.9% sequence similarity, respectively. The
sequence similarity between isolate 517 and both *H.*
halophilus, *H. miurensis*, and *G. halotolerans* was 93.4%.
Thus, the distance in 16S rRNA gene sequence similarity
between isolate 517 and the closest related is below the 97%
10 similarity, which is often used as a preliminary guideline
for species separation. A phylogenetic tree was created by
neighbour-joining analysis (bootstrap=100) using TREECON
1.3b software (Van de Peer and De Wachter, R. 1994) Fig. 1.

15 2.2 DNA-DNA hybridization and base composition analysis of
genomic DNA

DNA-DNA hybridization and DNA base composition (G+C
content) was carried out at DSMZ (Braunschweig, Germany).
20 DNA was isolated using a French pressure cell (Thermo
Spectronic) and was purified by chromatography on
hydroxyapatite as described by Cashion et al. (1977). DNA-
DNA hybridization was carried out as described by De Ley et
al. (1970) under consideration of the modifications
25 described by Huss et al. (1983) using a model Cary 100 Bio
UV/VIS-spectrophotometer equipped with a Peltier-
thermostatted 6x6 multicell changer and a temperature
controller with *in-situ* temperature probe (Varian). DNA-DNA
hybridization between isolate 517 and the closest related
30 based on 16S rRNA sequence similarity *P. ryukyuensis* was
28.8%, and between isolate 517 and *H. miurensis* it was
24.7%.

For determination of GC content, the DNA was hydrolyzed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (Mesbah et al. 1989) . The resulting deoxyribonucleotides were analyzed by HPLC. The DNA G+C content of isolate 517 was 38.4 mol%, which is fairly similar to the closest related species. The G+C content of *N. azotifigens* is 36.1-38.5 mol% (Sorokin et al. 2008), *H. halophilus* and *H. miurensis* is reported to be 38.5-40.7 mol% (Ishikawa et al. 2005), for *P. ryukyuensis* it is 35.6 mol% (Ishikawa et al. 2002), and for *G. halotolerans* it is reported to be 38 mol% (Wainø et al. 1999) .

The phylogenetic results and data on GC content indicate that isolate 517 represent a new species within a new genus, since the threshold value for DNA-DNA hybridization to separate two species is 70 % (Wayne et al., 1987). Thus, we propose that isolate 517 represents a new genus *Alkalilactibacillus* gen. nov. comprising the species *Alkalilactibacillus ikkense* sp. nov.

EXAMPLE 3

Characterization of native Ikka-beta-galactosidase from *Alkalilactobacillus ikkense*

3.1 Beta-galactosidase assay.

Beta-galactosidase activity was assayed by hydrolysis of o-nitrophenyl-beta-D-galactopyranoside (ONPG) and measuring the absorbancy of the released o-nitrophenyl (ONP) compound in a spectrophotometer at 415 nm. In the assay, the release

of ONP from 1 mM ONPG by the recombinant beta-galactosidase activity was measured at 415 nm at 20 °C and pH 7.0 (0.1 M NaH₂PO₄/Na₂HPO₄). The reactions were stopped by adding 300 µl 0.6 M Na₂CO₃. Assays were performed at 0, 5, 10, 20, 30, 5 40, 50, and 60 °C for 30 minutes. The sodium phosphate buffers were pre-heated to the respective temperatures prior to assay start.

Thermostability analysis of the enzyme was carried out by 10 placing an aliquot of enzyme at temperatures 0, 10, 20, 30, 40, and 50 °C and taking samples at t = 0 to t = 24 hours. Immediately after taking the samples, they were cooled and assayed at 20 °C as described above.

15 The pH activity profile was studied using a mixed pH buffer (250 mM Tris, 250 mM MES, 250 mM acetic acid) adjusted from pH 4 to pH 10 with HCl or NaOH. The samples were incubated at 20 °C for 1 hour and assayed as described above.

20 3.2 Production of native Ikka-beta-galactosidase.

Alkalilactibacillus ikkense cells were cultivated in liquid R2 medium supplemented with lactose and IPTG at 15 °C for 3 days on a rotary shaker. Cells were harvested by 25 centrifugation in a Sigma® 3-18M centrifuge at 4,700 rpm and the pellet was resuspended in 2 ml of 0.1 M NaH₂PO₄/Na₂HPO₄, pH 7. Cells were lysed by bead beating in a FastPrep FP120 instrument (Bio101/Savant) for 3 times 25 sec at speed 5.5. The supernatant was then removed from the 30 glass beads and centrifuged for 15 min at 10,000*g at 4 °C. The cell free supernatant was then used for assaying.

3.3 Characterization of temperature optimum of native Ikka-beta-galactosidase.

The native Ikka-beta-galactosidase displayed maximal activity at 20 °C, 40% of the maximal activity was obtained at 0 °C, and more than 60% of the maximal activity was observed at 10 °C (Fig. 2). Above 30 °C the enzyme was only moderately active and virtually no activity was observed at 60 °C.

The temperature stability of the Ikka-beta-galactosidase was investigated. Fig. 3 shows that almost 100% of the residual activity was observed after 24 hours incubation at 0 °C and that more than 80% activity was left after 24 hours at 20 °C. At temperatures above 20 °C, the native Ikka-beta-galactosidase rapidly lost activity (Fig. 3). The inactivation at high temperatures was shown to be irreversible.

The pH dependence of the native Ikka-beta-galactosidase was investigated. Fig. 4 shows that maximal activity of the native Ikka-beta-galactosidase was observed at pH 7, and that the enzyme displayed approximately 70% of the maximal activity at pH 6 and at pH 8. At pH 9, approximately 25% of maximal activity was observed. The enzyme showed no activity at pH 5 and below or at pH 10 and above (Fig. 4).

3.4 SDS-PAGE of native Ikka-beta-galactosidase.

Extracts from cells of *A. ikkense* induced with 1 mM IPTG and uninduced were analysed in SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) (Fig. 5). Intracellular extracts were prepared by lysing the cells

using bead-beating as described above in 3.2. The extracts (0.5-5 μ l) were mixed with 12.5 μ l 4*LDS sample buffer, 5 μ l 10*DTT and 0.1 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ to a final volume of 50 μ l. The samples were heated to 70 °C for 10 minutes and 30 μ l was loaded onto a 4-12% SDS gel. The gel was run in a XCell SureLock™ Mini-Cell (Invitrogen, CA, USA) at 150 V for 1 hour at room temperature. After electrophoresis, the gel was stained using Coomassie Brilliant Blue R-250 (0.1% Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) in 40% EtOH and 10% acetic acid). Fig. 5 shows that a strong 120 kDa band was observed in the lanes with extracts from *A. ikkense* cells induced with IPTG and that this band was missing in lanes with extracts from non-induced cells. Thus, the 120 kDa band was assumed to be the native Ikka-beta-galactosidase.

EXAMPLE 4

Isolation and characterization of the Ikka-beta-galactosidase gene from *Alkalilactobacillus ikkense*

4.1 Isolation of the Ikka-beta-galactosidase gene

DNA from *A. ikkense* was isolated from a culture of 50 ml. The cells were harvested by centrifugation and the chromosomal DNA was isolated using conventional phenol-chloroform extraction methods (Maniatis et al., 1982). The DNA was partially digested using *Sau*3AI (New England Biolabs, MA, USA), and fragments with the lengths between 3 kb and 10 kb were purified from an agarose gel using the

QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) as described by the producer.

The vector for cloning chromosomal DNA from *A. ikkense* was
5 a modified pUC18 plasmid (Stratagene, CA, USA). Plasmid
pUC18 was restricted by *Nde*I and *Hind*III endonucleases (New
England Biolabs). The sticky ends were refilled using
Klenow fragment of DNA polymerase (New England Biolabs) and
the blunt ends were ligated using T4 DNA ligase (New
10 England Biolabs). DNA sequencing of the modified pUC18
plasmids, denoted pUC18dlacZ, at GATC Biotech AG (Konstanz,
Germany) and analysis of the DNA sequence in the CLC
Workbench 4 software (CLC bio, Aarhus, Denmark) confirmed
that the α -subunit sequence of pUC18 was deleted in plasmid
15 pUC18dlacZ. Thus, plasmid pUC18dlacZ was not able to
mediate α -complementation when introduced into *E. coli*
cells harbouring the beta-galactosidase Δ Z15 mutation.

*Sau*3AI restricted and gel-purified chromosomal DNA from *A.*
20 *ikkense* was ligated into plasmid pUC18dlacZ treated with the
restriction endonuclease *Bam*HI and Antarctic Phosphatase (New
England Biolabs). The ligation mixture was transformed into
chemically competent *E. coli* TOP10 cells. Transformed cells
were plated onto LB agar (10 g/l Bacto tryptone, 5 g/l yeast
25 extract, 10 g/l NaCl) containing 20 μ g/ml X-gal, 0.1 mM IPTG,
and 100 μ g/ml ampicillin and incubated over night at 37 °C.
After a 16 hours over night incubation, the plates were
transferred to 20 °C and incubated for another 20 hours. A
total of 580 colonies were screened and 1 blue colony was
30 detected. The colony that turned blue during incubation at 20
°C was selected and transferred to 10 ml LB broth and grown
at 37 °C over night. Recombinant *E. coli* cells from over
night cultures were harvested by centrifugation, and plasmid

DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNA was analysed for inserts by digestion with restriction endonucleases *EcoRI* and *PstI* (New England Biolabs). The insert in the plasmid, denoted pUCIkka-bgal, was sequenced at GATC Biotech AG (Konstanz, Germany) using primer walking with the primer M13 reverse and custom made primers specific for the insert in pUCIkka-bgal (SEQ ID 3: 5'CCGTCATCCATATCACCC3'; SEQ ID 4: 5'CCTTTGCCCAAGAGCCAACC3'; SEQ ID 5: 5'GCTATTATCAGACTTGGCACCC3'; SEQ ID 6: 5'GTAATTCAAT GTTCCAACGTG3'; Seq ID 7: 5'CGCTTATGGTGTGAAG3') and a sequence just downstream of the multiple cloning site in pUC18dlacZ, (SEQ ID 8: 5'GGGCTGGCTTAACTATGCGG3'). The Ikka-beta-galactosidase gene sequence harboured by the DNA insert is shown as SEQ ID NO 2.

4.2 Characterization of the Ikka-beta-galactosidase gene sequence

Analysis of the DNA sequence, SEQ ID NO 2, using the CLC Workbench 4 software (CLC bio, Aarhus, Denmark) showed an open reading frame with the coding capacity of 1,041 amino acids, SEQ ID NO 1. The NCBI search tool Blastp was used to search for related sequences in databases. The closest related sequences were beta-galactosidases from *Bacillus megaterium* (accession no ABN13675) 56.7% identity, *Paenibacillus* sp. JDR-2 (accession no ZP_02849115) 55.3% identity, and *Geobacillus* sp. Y412MC10 (accession no ZP_03036811) 54% identity, all of which belong to the Glycosyl Hydrolase Family 2. Thus, it is concluded that the Ikka-beta-galactosidase belongs to this family. The calculated subunit molecular weight and pI of the Ikka-beta-galactosidase was 119 kDa and pI 5.0, respectively (ExPASy ProtParam tool). The calculated subunit molecular

weight was confirmed by SDS-PAGE, Figs. 5 and 6. Alignment of the Ikka-beta-galactosidase with structurally resolved enzymes showed that the conserved active site region in *E. coli* (ILCEYAHAMGN) (pos. 534-544) (Gebler *et al.* 1992) is well conserved in the Ikka-beta-galactosidase (ILCEFSHAMGN) (pos. 547-557), and the active site nucleophile Glu-537 is probably found as Glu-550.

10 EXAMPLE 5

Production of recombinant Ikka-beta-galactosidase in *Escherichia coli*.

15 Native *Alkalilactibacillus ikkense* was shown to produce only moderate amounts of Ikka-beta-galactosidase. Therefore, in order to produce larger amounts of the beta-galactosidase, subcloning of the Ikka-beta-galactosidase gene into expression plasmids was carried out.

20

5.1 Construction of a vector for the expression of recombinant Ikka-beta-galactosidase in *Escherichia coli*.

The Ikka-beta-galactosidase gene was subcloned further using chromosomal DNA from *A. ikkense* as template and the PCR primers bGal5':

5'CTGAATTCGCATATGGCAAAAAAATTAAAAAATTC3' (*Eco*RI restriction site underlined) (SEQ ID 9), and bGal3':

5'CCAAGCTTATCTGTTTAACTATTCAACATG3' (*Hind*III site double underlined) (SEQ ID 10). The polymerase used was the proofreading polymerase Phusion[®] High-Fidelity DNA Polymerase (New England BioLabs). The PCR reaction was analyzed by gel electrophoresis on a 0.8% agarose gel

(Seakem GTG) and the 3.9 kb fragment was ligated into pJET1.2/blunt cloning vector (Fermentas, Helsingborg, Sweden) and transformed into *E. coli* TOP10 cells. *E. coli* transformants containing pJET1.2/blunt were isolated on
5 ampicillin containing LB agar plates and plasmid DNA was prepared as described above. Plasmid DNA was restricted with the enzymes *EcoRI* and *HindIII* and analysed on 0.8% (w/v) agarose gels as described. The 3.9 kb DNA fragment
10 was purified from the gel using the QIAquick Gel Extraction Kit as described by the producer. The purified DNA fragment was ligated into plasmid pUC18dlacZ similar restricted with the enzymes *EcoRI* and *HindIII* and gel purified as described above. The ligation mixture was transformed into *E. coli* TOP10 cells and recombinant cells harbouring the plasmid
15 pUC18dlacZ with the Ikka-beta-galactosidase gene were selected as blue colonies on LA plates containing 100 µg/ml ampicillin, 1 mM IPTG, and 40 µg/ml X-gal. Transformants were selected and analysed for plasmids and inserts. Plasmid DNA was prepared from a 10 ml culture and the DNA
20 was sent for sequencing at GATC biotech (Konstanz, Germany) using the primers described above in 4.1. The entire Ikka-beta-galactosidase gene was sequenced on both strands in order to ensure that no mutations were introduced during PCR. One of the recombinant clones, which contained plasmid
25 pUC18dlacZ with the Ikka-beta-galactosidase gene, denoted plasmid pUCIkka-bgal_exp, was selected for further expression studies.

30 5.2 Expression of recombinant Ikka-beta-galactosidase in *Escherichia coli*.

E. coli TOP10 cells harbouring plasmid pUCIkka-bgal and pUCIkka-bgal_exp were cultivated in 30 ml LB broth

containing 100 µg/ml ampicillin over night at 37 °C. After an over night incubation, the cells were supplemented with 0.1 mM IPTG and incubated at 20 °C for further 20 hours. Cells were harvested by centrifugation for 30 min at 4,700 rpm at 10 °C and resuspended in 1 ml 0.1 M NaH₂PO₄/Na₂HPO₄, pH 7. The cells were lysed by bead beating in a Fast Prep instrument (Fast Prep FP120, Bio101/Savant Instruments Inc., Holbrook, NY) at speed 5.5 for 3 times 25 sek. The samples were cooled on ice in between the beating/shaking. The lysate was centrifuged at 10,000 g for 15 min at 4 °C, and the supernatant containing the Ikka-beta-galactosidase enzyme was transferred to a clean tube. This crude extract was used for subsequent analyses.

5.3 Properties of recombinant Ikka-beta-galactosidase produced in *Escherichia coli*.

5.3.1. SDS-PAGE of recombinant Ikka-beta-galactosidase and determination of yield produced.

Intracellular extracts from recombinant *E. coli* cells harbouring plasmid pUCIkka-bgal were analysed on SDS-PAGE (SDS gel 4-12%, PAGEgel, CA, USA) as described above in 3.4. Cultures induced with 1 mM IPTG and control cultures not induced were analysed.

The protein bands in extracts from cultures grown with and without IPTG were identical apart from a band of approximately 120 kDa in cultures induced with IPTG (arrow in Fig. 6). Thus, as the calculated molecular mass of the Ikka-beta-galactosidase is 119 kDa, and since the strong band at 120 kDa was observed only in cultures induced with

IPTG, it is assumed that the 120 kDa band represent the Ikka-beta-galactosidase.

Extracts from cultures of plasmid pUCIkka-bgal harbouring
5 *E. coli* were prepared as described above and diluted before electrophoresis on SDS-PAGE. Beta-galactosidases from *E. coli* (Sigma-Aldrich, MO, USA) and *K. lactis* (Novozymes, Bagsvaerd, Denmark) with known molecular mass and in defined concentrations were co-electrophorezed on the same
10 gel for comparison (arrows in Fig. 6). By comparing the migration and Coomassie Brilliant Blue staining of the known beta-galactosidases with that of the Ikka-beta-galactosidase an estimate of the amount of the Ikka-beta-galactosidase was obtained. The extract, which was used for
15 the subsequent analyses was estimated to have a concentration of Ikka-beta-galactosidase of 2 mg/ml.

5.3.2 Temperature dependence of recombinant Ikka-beta-galactosidase.

20

The temperature optimum of recombinant Ikka-beta-galactosidase was determined as described above for the native enzyme using ONPG as a substrate. The temperature profile was determined at 0, 5, 10, 20, 30, 40, 50, and 60
25 °C for 30, 60 and 120 minutes for the recombinant Ikka-beta-galactosidase and, as controls, for the beta-galactosidases from *E. coli* and *K. lactis*. The optimal temperature for activity of the recombinant Ikka-beta-galactosidase was determined to be 20-30 °C (Fig. 7).
30 However, the Ikka-beta-galactosidase also showed high activity at low temperatures with more than 40% activity at 0 °C, approximately 80% activity at 5 °C and more than 90% at 10 °C. Compared to the *K. lactis* beta-galactosidase, the

specific activity of the Ikka-beta-galactosidase was almost twice as high at temperatures between 0 °C and 30 °C (Fig. 8). Both enzymes showed close to zero activity at 40 °C and above (Fig. 8).

5

The thermostability of the Ikka-beta-galactosidase was determined at 0, 10, 20, 30, 40, 50, and 60 °C. Samples were taken from t = 0 hours to t = 123 hours with increasing intervals from 5 minutes during the first hour to several hours at the end of the experiment (Fig. 9). Figs. 9 and 10 show that the Ikka-beta-galactosidase showed high stability at 0 °C and 5 °C, at 10 °C the enzyme was stable for approximately 100 hours and at temperatures above 20 °C the Ikka-beta-galactosidase was rather unstable. Treatment at 40 °C for 40 minutes resulted in complete inactivation. The inactivation of the Ikka-enzyme was irreversible.

5.3.3 pH dependence of recombinant Ikka-beta-galactosidase.

20

The pH activity profile was studied using a mixed pH buffer (250 mM Tris, 250 mM MES, 250 mM acetic acid) adjusted from pH 4 to pH 10 with HCl or NaOH. The samples were incubated at 20 °C for 2 hours. The optimal pH value for the Ikka-beta-galactosidase was shown to be approximately pH 7.0. At pH 6.0 the enzyme showed 60% of maximal activity and at pH 8.0 the Ikka-enzyme displayed 90% activity. At pH 9.0 15% activity was observed whereas no activity could be detected at pH 5.0 or below or at pH 10 and above.

30

5.3.3 pH dependence of recombinant Ikka-beta-galactosidase.

The substrate specificity of the Ikka-beta-galactosidase
5 was determined in assays performed at pH 7.0 and 20 °C for
20 minutes using nine different chromogenic substrates, o-
nitrophenyl-beta-D-galactopyranoside, p-nitrophenyl-alpha-
D-galactopyranoside, o-nitrophenyl-beta-D-glucopyranoside,
p-nitrophenyl-beta-D-glucopyranoside, p-nitrophenyl-beta-D-
10 arabinopyranoside, p-nitrophenyl-beta-D-cellobioside, p-
nitrophenyl-beta-D-fucopyranoside, p-nitrophenyl-beta-D-
lactopyranoside, and p-nitrophenyl-beta-D-mannopyranoside.
Each substrate was used at a concentration of 10 mM. The
assays showed that the Ikka-beta-galactosidase was only
15 able to hydrolyze o-nitrophenyl-beta-D-galactopyranoside
(ONPG) and p-nitrophenyl-beta-D-fucopyranoside (4 % of the
relative activity compared to hydrolysis of ONPG). The
utilization of the remaining substrates was below
detection.

20 Hydrolysis of lactose was determined in a solution of
lactose in water. Three different lactose concentrations
were tested: 1.25 mg/ml, 2.5 mg/ml, and 5 mg/ml. Total
reaction volume was 0.2 ml and each reaction contained 0.2
25 mg/ml of recombinant Ikka-beta-galactosidase enzyme. The
enzyme reactions were incubated at 5 °C and 20 °C, and
samples were collected after 15 minutes, 2½ hour, and 24
hours. After incubation, the reactions were stopped by
heating at 95 °C for 20 minutes. Visualization of the
30 products was carried out by thin-layer chromatography (TLC)
on a TLC Silica gel 60 (Merck, Darmstadt, Germany) in a
solvent containing 1-butanol, 2-propanol, and water
(3:12:4). Volumes containing 0.005 mg lactose were run on

the TLC. Controls were 0.5 µl lactose (2.5 %), 0.5 µl galactose (2.5 %) and 0.5 µl glucose (2.5 %). After being dried, the sugars were visualized by spraying with an orcinol reagent followed by incubation for 5-10 min at 100 °C.

Hydrolysis of lactose was observed both at 5 °C and at 20 °C. At 5 °C, approximately 75-85% lactose was hydrolysed in the 1.25 mg/ml reaction after 15 minutes and 100% was hydrolysed within 2½ hours, (Fig. 12 A). Similar hydrolysis efficiency was observed in the 1.25 mg/ml reaction incubated at 20 °C (Fig. 12 B). Hydrolysis effectiveness in the 2.5 mg/ml lactose reaction showed approximately 90-95% hydrolysis within 2½ hours at both temperatures. After 24 hours, 100% hydrolysis was observed for all three lactose concentrations at both temperatures (Fig. 12).

5.3.4 Purification of recombinant Ikka-beta-galactosidase.

20

Beta-galactosidase was purified from crude extracts by ion exchange chromatography. Portions of two ml were subjected to chromatography on a 1 ml High Q* cartridge on a BioLogic LP System (Bio-Rad).

25

The column was washed with 10 ml of 50 mM phosphate buffer (pH 7) and eluted by a gradient from 0 to 1 M of NaCl in 50 mM phosphate buffer (pH 7) at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected. Crude extracts were also subjected to affinity chromatography on a 2 ml column of agarose coupled with p-aminobenzyl-1-thio-beta-D-galactopyranoside (PABTG-agarose, Sigma). The column was washed with 10 ml of 50 mM phosphate buffer (pH 7) before

30

* Trademark

it was eluted by 100 mM NaCl in 50 mM phosphate buffer (pH 7) at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected.

5 The fractions were analysed for the presence of proteins using a BCA Protein Assay Kit (Pierce), and beta-galactosidase was measured in o-nitrophenyl (ONP)-beta-D-galactopyranoside assays as described above. Fractions containing beta-galactosidase activity were analysed by SDS
10 polyacrylamide gel electrophoresis (4-20 %, PAGEgel, CA, USA). Purified beta-galactosidase was used for subsequent stability and activity experiments.

Fig. 13 shows that the crude extract from *E. coli* contained
15 recombinant beta-galactosidase with a monomeric molecular weight of approximately 115-120 kDa. Ion exchange chromatography resulted in pure beta-galactosidase (Fig. 13, lane 2), whereas affinity chromatography (Fig. 13, lane 3) only resulted in partially purified recombinant enzyme.
20 Thus, for the subsequent analyses, pure beta-galactosidase from ion exchange was used, unless otherwise specified.

5.3.5 Characterization of native and recombinant *A. ikkense* beta-galactosidase.

25

The molecular weight of the *A. ikkense* beta-galactosidase was determined to be approximately 115-120 kDa when analyzed on SDS-PAGE using known beta-galactosidases from *E. coli* and *K. lactis* as references. This result is in
30 agreement with the calculated molecular weight as determined from the DNA sequence (119 kDa). The crude extract from *E. coli* was estimated to contain 10 mg/ml *A. ikkense* beta-galactosidase.

The specific activity, calculated on the basis of purified beta-galactosidase from ion exchange chromatography was 8.4 micromoles/min/mg protein at 20 °C with ONPG as substrate

5 (Table 1).

Table 1

Purification	Volume (ml)	Protein (mg)	Specific activity (U mg ⁻¹)	Total activity (U)	Purification (fold)	Recovery (%)
Cell extract	10	30	1.6	48.0	1	100
Ion exchange	10	2.5	8.4	21.1	12	44

10 A culture of 200 ml *E. coli* cells expressing recombinant *A. ikkense* beta-galactosidase was harvested resulting in a 280 mg wet weight cell pellet. The cells were lyzed in a Fast Prep apparatus, and the extract was subjected to ion exchange chromatography. One U is 1 micromole per min at 20 °C with ONPG as substrate.b

15 Extract of *E. coli* expressing recombinant *A. ikkense* beta-galactosidase was analyzed for beta-galactosidase activity.

20 The thermal stability of the purified, recombinant *A. ikkense* beta-galactosidase enzyme was tested as shown in Figure 15. An equal amount of enzyme was incubated at the temperatures indicated and samples were withdrawn at different time intervals. Y-axis is the residual activity in percent of maximal activity. Enzyme samples were incubated at temperatures from 0 °C to 60 °C and at the time points indicated (X-axis), samples were withdrawn and
25 assayed for active beta-galactosidase at 20 °C. Assays were performed in triplicates, and standard error was below 0.05.

30 At 0 °C the enzyme displayed more than 60 % of the maximal activity, and at 10 °C, more than 70 % of the maximal activity was observed both for purified recombinant enzyme.

Analysis of enzyme stability showed that purified, recombinant beta-galactosidase was 100% stable at 0 °C to 20 °C for at least 5 hours (Fig. 15), and that the residual activity after 5 days storage at 0 °C to 20 °C was 50-60 % (data not shown). At 30 °C, the purified beta-galactosidase lost more than 80 % of its activity within 5 hours. Complete, irreversible inactivation was achieved within 5 minutes at 50 °C and within 10 minutes at 40 °C (Fig. 15).

10 The Temperature dependence (A) and pH dependence (B) of the purified, recombinant *A. ikkense* beta-galactosidase was tested (Figure 14). Y-axis is the relative activity in percent of maximal activity. X-axis is the incubation temperature (A) or pH (B). The relative activity of the purified, recombinant enzyme with ONPG as substrate is illustrated by black squares. Assays were performed in triplicates, and standard error was below 0.05.

20 Maximal activity of the purified, recombinant enzyme was observed at pH 8 (Fig. 14B). About 60 % of the maximal activity was maintained at pH 7, and at pH 9 approximately 90 % activity was observed (Fig. 14B).

The recombinant *A. ikkense* beta-galactosidase was benchmarked with Lactozyme 3000® 13 from *K. lactis*. At temperatures between 0 °C and 20 °C the *A. ikkense* beta-galactosidase showed a twofold increase in conversion rate, when compared to the *K. lactis*-beta-galactosidase (Fig. 16); *A. ikkense* beta-galactosidase (black squares) compared with commercially available Lactozyme® 3000L from *K. lactis* (open circles). Specifically the experiment was conducted in the following way: An equal amount of enzyme (2 mg/ml) was incubated with ONPG as substrate at temperatures from 0 °C

to 20 °C. Samples were withdrawn at different time intervals and hydrolyzed ONP was measured at A420 nm. Hydrolysis efficiency was calculated as increase in A420 nm per min per microgram active enzyme. Assays were performed
5 in triplicates, and standard error was below 0.05.

Investigation of substrate specificity of the *A. ikkense* β -galactosidase was carried out using nine different chromogenic substrates. Hydrolysis was only observed with
10 ONPG and with *p*-nitrophenyl-beta-D-fucopyranoside (4 % of the relative activity compared to hydrolysis of ONPG). Thin-layer chromatography (TLC) was used to demonstrate the hydrolysis of lactose by the *A. ikkense* beta-galactosidase (Figure 17). Lanes 4-6: samples incubated at 5 °C for 2½ h
15 (4: 1.25 mg/ml lactose, 5: 2.5 mg/ml lactose, 6: 5 mg/ml lactose). Lanes 7-9: samples incubated at 20 °C for 2½ h (7: 1.25 mg/ml lactose, 8: 2.5 mg/ml lactose, 9: 5 mg/ml lactose). Lanes 1-3: Controls, 0.0125 mg of each of the carbohydrates lactose (lane 1), galactose (lane 2) and
20 glucose (lane 3).

Hydrolysis of the remaining substrates was below detection limit. Hydrolysis of lactose was observed at both 5 °C and at 20 °C (Fig. 17). At 5 °C, approximately 75-85 % of the
25 lactose was hydrolyzed in the 1.25 mg/ml reaction after 15 minutes, and 100 % of the lactose was hydrolyzed within 2½ hours (Fig. 17, lane 4). Similar hydrolysis efficiency was observed in the 1.25 mg/ml reaction incubated at 20 °C for 2½ hours (Fig. 17, lane 7). Hydrolysis effectiveness in the
30 2.5 mg/ml lactose reaction showed approximately 90-95 % hydrolysis within 2½ hours at both temperatures (Fig. 17, lanes 5 and 8). After 24 hours, 100 % hydrolysis was observed for all three lactose concentrations at both

temperatures (not shown). At the highest lactose concentration (27 mg/ml), the TLC gel indicated the formation of oligosaccharides (Fig. 17, lanes 6 and 9).

5

EXAMPLE 6

Production of recombinant Ikka-beta-galactosidase in *Bacillus subtilis*.

- 10 The Ikka-beta-galactosidase was further subcloned in a *Bacillus subtilis* expression vector, pAL10 (MoBiTech, GmbH). PCR was performed using chromosomal DNA from *Alkalilactibacillus ikkense* as template and the PCR primers Bs_pAL_bGal5': 5'GGCCATGGATCCATGGCAAAAAAATTAAAAAATTC3' (BamHI restriction site underlined) (SEQ ID NO 11) and
- 15 Bs_pAL_bGal3': 5'GGCCATCCCGGGTTATCTGTTTAACTATTCAACATG3' (XmaI restriction site double underlined) (SEQ ID NO 12). PCR, subsequent isolation of the fragment encoding the Ikka-beta-galactosidase, ligation into pUC18dLacZ and
- 20 transformation of *E. coli* was as described above in 5.1. Plasmid pUC18dLacZ carrying the Ikka-beta-galactosidase gene was prepared and sequenced, before the plasmid DNA was restricted with restriction endonucleases *Bam*HI and *Xma*I. The 3.1 kb fragment encoding Ikka-beta-galactosidase was
- 25 purified, inserted into plasmid pAL10 similarly restricted with *Bam*HI and *Xma*I, and transformed into *E. coli* as described in 5.1. Recombinant *E. coli* harbouring pAL10 containing the Ikka-beta-galactosidase gene was isolated on LB agar plates containing 100 µg/ml ampicillin. Plasmid
- 30 pAL10_Ikka-bGal was purified and transformed into *B. subtilis* cells using an electroporation protocol for *B. subtilis* from Eppendorf (Germany) (Protocol No. 4308 915.504 - 08/2003). Recombinant cells harbouring plasmid

pAL10 were selected on LB agar containing 5 µg/ml chloramphenicol.

Production of recombinant Ikka-beta-galactosidase in *B. subtilis* was carried out by growing *B. subtilis* pAL10_Ikka-bGal cells in LB containing 5 µg/ml of chloramphenicol at 37 °C for 16 hours. Induction of Ikka-beta-galactosidase synthesis was carried out by changing the temperature to 20 °C. *B. subtilis* pAL10_Ikka-bGal cells were cultivated at 20 °C for another 5 hours after which, the cells were harvested, and intracellular enzyme was isolated by Fast Prep as described in 5.2.

A crude, intracellular extract from *B. subtilis* pAL10_Ikka-bGal cells was analysed in ONPG assays as described in 5.3. ONPG assays showed the presence of a cold-active Ikka-beta-galactosidase with an activity similar to the native enzyme, to the recombinant enzyme in crude *E. coli* extracts, and to the pure enzyme produced in *E. coli* cells.

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30

SEQ ID NO: 1

(1) GENERAL INFORMATION:

5 (i) APPLICANT:

(A) NAME: University of Copenhagen

(B) STREET:

(C) CITY:

(D) COUNTRY:

10 (E) POSTAL CODE:

(ii) TITLE OF INVENTION: Cold-active beta-galactosidase,
a method of producing same and use of such enzyme

15 (iii) NUMBER OF SEQUENCES: 10

(2) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 1,041 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: Not relevant

(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION SEQ ID NO: 1

1 10
30 Met Ala Lys Lys Leu Lys Lys Phe Asn Tyr Leu Pro Pro Lys Asn
20 30
Gly Tyr Pro Glu Trp Asn Asn Asn Pro Glu Ile Phe Gln Leu Asn
35 40
Arg Arg Glu Ala His Ala Thr Leu Val Pro Tyr Ser Asn Leu Glu
50 60
Leu Ala Leu Lys Gly Glu Arg Thr Ala Ser Ser Phe Tyr Gln Ser
40
70
Leu Asn Gly Ser Trp Gln Phe Ala Phe Ala Gln Glu Pro Thr Lys
80 90
45 Arg Val Ile Asp Phe Tyr Arg Lys Asp Phe Asp His Arg Asp Trp

100

	Asp	Ser	Ile	Lys	Val	Pro	Ser	His	Trp	Gln	Leu	Glu	Gly	Tyr	Asp
					110										120
5	Tyr	Pro	Gln	Tyr	Thr	Asn	Thr	Thr	Tyr	Pro	Trp	Val	Glu	Lys	Glu
										130					
	Thr	Ile	Lys	Pro	Pro	Phe	Ala	Pro	Thr	Asn	Tyr	Asn	Pro	Val	Gly
					140										150
10	Gln	Tyr	Val	Arg	Thr	Phe	Glu	Leu	Pro	Thr	Asp	Trp	Asn	Gly	Ala
										160					
	Pro	Val	Tyr	Leu	Asn	Phe	Gln	Gly	Val	Glu	Ser	Ala	Phe	Tyr	Val
15					170										180
	Trp	Ile	Asn	Gly	Asp	Leu	Val	Gly	Tyr	Ser	Glu	Asp	Thr	Phe	Thr
										190					
20	Pro	Ala	Glu	Phe	Asp	Ile	Thr	Pro	Tyr	Leu	Ile	Glu	Gly	Glu	Asn
					200										210
	Lys	Leu	Ala	Val	Glu	Val	Tyr	Arg	Trp	Ser	Asp	Ala	Ser	Trp	Leu
										220					
25	Glu	Asp	Gln	Asp	Phe	Trp	Arg	Leu	Ser	Gly	Ile	Phe	Arg	Asp	Val
					230										240
	Tyr	Leu	Tyr	Ala	Thr	Pro	Ala	Gln	His	Ile	Asp	Asp	Phe	Phe	Val
30										250					
	Thr	His	Glu	Leu	Asp	Ala	Asp	Tyr	Arg	Asn	Ala	Thr	Leu	Lys	Ile
					260										270
35	Asp	Met	Lys	Val	Arg	Asp	Tyr	Phe	Glu	Ile	Gly	Glu	Pro	Val	Thr
										280					
	Val	Asn	Ala	Met	Leu	Phe	Asp	Leu	Asn	Gly	Asn	Pro	Val	Leu	Lys
					290										300
40	Gln	Pro	Leu	Leu	Ser	Ala	Val	Asp	Phe	Ser	Gly	Lys	Glu	Val	Ala
										310					
	Asp	Val	Ser	Val	Ile	Thr	Thr	Ile	Asp	Asn	Pro	Leu	Lys	Trp	Ser
45					320										330
	Ala	Glu	Asp	Pro	Asn	Leu	Tyr	Thr	Leu	Val	Leu	Ser	Leu	Val	Asp

Gln Asn Gly Lys Leu Leu Glu Thr Glu Ser Cys Arg Val Gly Phe
 350 360
 Arg Lys Phe Glu Arg Lys Asp Gly Leu Met Gln Ile Asn Gly Lys
 5
 370
 Arg Ile Val Phe Lys Gly Thr Asn Arg His Glu Phe Ala Ser Asp
 380 390
 10 Lys Gly Arg Ala Ile Thr Ile Asp Asp Met Val Asn Asp Ile Gln
 400
 Leu Met Lys Gln His Asn Ile Asn Ala Val Arg Thr Ser His Tyr
 15 410 420
 Pro Asn His Pro Leu Trp Tyr Glu Leu Cys Asp Thr Tyr Gly Leu
 430
 Tyr Val Ile Asp Glu Thr Asn Leu Glu Thr His Gly Thr Trp Val
 20 440 450
 Tyr Gly Gln Lys Gly Leu Ala Glu Thr Ile Pro Gly Ser Leu Pro
 460
 25 Lys Trp Thr Glu Asn Val Leu Asp Arg Cys Asn Ser Met Phe Gln
 470 480
 Arg Asp Lys Asn His Pro Ser Ile Leu Asp Trp Ser Leu Gly Asn
 30 490
 Glu Ser Phe Gly Gly Asp Asn Phe Leu Lys Met His Asp Phe Phe
 500 510
 Thr Glu Gln Asp Pro Ala Arg Leu Val His Tyr Glu Gly Ile Phe
 35 520
 His Tyr Arg Glu Ser Glu Arg Ala Ser Asp Met Glu Ser Thr Met
 530 540
 40 Tyr Ile Ser Pro Glu Gly Ile Glu Asp Tyr Ala Lys Lys Ala Thr
 550
 Lys Glu Thr Lys Pro Tyr Ile Leu Cys Glu Phe Ser His Ala Met
 45 560 570
 Gly Asn Ser Leu Gly Asn Phe Tyr Lys Tyr Thr Glu Leu Phe Asp

	Gln	Tyr	Pro	Ile	Leu	Gln	Gly	Gly	Phe	Ile	Trp	Asp	Trp	Lys	Asp
					590										600
5	Gln	Ser	Leu	Leu	Thr	Lys	Thr	Ala	Gly	Gly	Thr	Pro	Tyr	Leu	Ala
										610					
	Tyr	Gly	Gly	Asp	Phe	Gly	Glu	Ser	Pro	His	Asp	Gly	Asn	Phe	Ala
					620										630
10	Gly	Asn	Gly	Leu	Ile	Phe	Gly	Asp	Gly	Lys	Val	Ser	Pro	Lys	Ile
										640					
	Phe	Glu	Val	Lys	Arg	Cys	Tyr	Gln	Asn	Val	Asp	Phe	Lys	Ala	Ile
15					650										660
	Asp	Leu	Val	His	Gly	Gln	Ile	Glu	Leu	Thr	Asn	Lys	Tyr	Leu	Phe
										670					
20	Thr	Asn	Leu	Ala	Asp	Tyr	Gln	Leu	Asn	Trp	Val	Ile	Thr	Arg	Asn
					680										690
	Gly	Asp	Ala	Ile	Glu	Ser	Gly	Ala	Thr	Asn	Ile	Asn	Val	Leu	Pro
										700					
25	Gly	Glu	Lys	Arg	Glu	Val	Ile	Leu	Asp	Tyr	Thr	Phe	Pro	Thr	Gly
					710										720
	Val	Cys	Met	Thr	Asp	Glu	Tyr	Ile	Leu	Thr	Leu	Arg	Phe	Ser	Glu
30										730					
	Lys	Gly	Asp	Arg	Leu	Trp	Cys	Glu	Ala	Gly	His	Glu	Val	Ala	Phe
					740										750
35	Asn	Gln	Phe	Val	Leu	Pro	Thr	Lys	Val	Thr	Lys	Leu	Arg	Glu	Lys
										760					
	Thr	Gln	Asp	Thr	Lys	Thr	Leu	Ser	Val	Glu	Val	Met	Gln	Asp	Arg
					770										780
40	Leu	Val	Thr	Ser	Gly	Ala	Gly	Phe	Ser	Val	Gly	Phe	Asp	Thr	Lys
										790					
	Ser	Gly	Met	Leu	Val	Ser	Tyr	Gln	Val	Gly	Gly	Asn	Glu	Leu	Val
45					800										810
	Lys	Glu	Ala	Leu	Val	Pro	Asn	Phe	Trp	Arg	Ala	Met	Thr	Asp	Asn

Asp Arg Gly Asn Gly Leu Asp Gln Arg Ser Gln Ile Trp Arg Asp
 830 840
 5 Ala Asn Glu Val Arg Glu Leu Val Ser Phe Gln Tyr Glu Val Leu
 850
 Thr Asn Arg Val Ser Ile Ser Thr Val Phe Leu Tyr Glu Asp Leu
 860 870
 10 Asn His Ser Arg Val Glu Leu Asn Phe Leu Ile Thr Gly Thr Gly
 880
 Glu Ile Lys Val Asp Tyr Val Leu Lys Pro Gly Glu Asp Leu Pro
 890 900
 15 Glu Ile Pro Glu Ile Gly Leu Met Leu Thr Met Pro Lys Ser Phe
 910
 20 Asp Gln Leu Ser Trp Tyr Gly Lys Gly Pro His Glu Ser Tyr Trp
 920 930
 Asp Lys Gln Lys Gly Ala Lys Ile Gly Leu Tyr Gln Gly Phe Val
 940
 25 Gly Asp Gln Tyr Val Pro Tyr Leu Lys Pro Gln Glu Cys Gly Asn
 950 960
 Lys Val Gly Val Arg Ser Ala Glu Leu Val Asn Asp Val Gly Val
 970
 30 Gly Leu Ile Ile Ser Gly Leu Pro Thr Leu Glu Leu Asn Val Leu
 980 990
 35 Pro Tyr Thr Pro Val Gln Leu Glu Ser Ala Asp His Ser Tyr Gln
 1000
 Leu Pro Glu Thr Asp Gln Thr Val Val Arg Ile Asn Leu Gly Gln
 1010 1020
 40 Met Gly Val Gly Gly Asp Asp Ser Trp Gly Gln Arg Thr His Gln
 1030
 Asp Phe Thr Leu Phe Ala Asn Lys Thr Tyr His Tyr Ser Phe Met
 1040
 45 Leu Asn Ser Leu Asn Arg

SEQ ID NO: 2

(2) INFORMATION FOR SEQ ID NO: 2

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3,123nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY:linear

10

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION SEQ ID NO: 2

15

ATGGCAAAAA AATTAAAAAA ATTCAACTAC CTCCCACCAA AAAACGGGTA 50

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20

CGCATGCAAC ATTGGTGCCA TATTCTAATT TGGAATTGGC ACTTAAAGGG 150

GAGCGGACAG CATCATCATT TTATCAATCT TTAAATGGTA GTTGGCAGTT 200

TGCCTTTGCC CAAGAGCCAA CCAAGCGAGT GATAGATTTT TATCGGAAAG 250

25

ATTTTGATCA TCGCGATTGG GATTCGATTA AAGTACCAAG TCATTGGCAG 300

TTAGAAGGCT ATGACTACCC GCAATACACC AACACAACGT ACCCATGGGT 350

30

AGAAAAAGAA ACGATTAAAC CTCCATTTGC ACCAACAAAT TATAATCCAG 400

TCGGACAATA TGTTTCGCACG TTTGAATTAC CGACTGATTG GAATGGAGCT 450

CCCGTTTATC TGAATTTCCA AGGTGTTGAA TCAGCTTTCT ACGTCTGGAT 500

35

AAATGGTGAT TTGGTCGGAT ACAGTGAGGA CACTTTCACA CCAGCTGAAT 550

TTGATATAAC TCCCTATTTA ATAGAGGGTG AAAATAAGCT AGCGGTAGAA 600

40

GTCTATCGTT GGAGTGATGC GAGCTGGCTT GAAGACCAGG ATTTCTGGAG 650

GTTAAGCGGG ATTTTTCGTG ACGTCTATCT ATATGCAACA CCAGCTCAGC 700

ACATTGATGA TTTCTTTGTC ACACACGAAC TTGATGCAGA CTATCGAAAT 750

45

GCAACGTTGA AGATTGATAT GAAAGTGCGC GATTATTTCG AGATTGGCGA 800

GCCTGTCACA GTTAATGCGA TGCTCTTTGA TCTTAATGGG AATCCGGTTC 850

	TCAAGCAACC	GCTTTTATCG	GCAGTAGATT	TTTCAGGTAA	AGAAGTTGCT	900
	GATGTGAGCG	TAATAACAAC	AATTGATAAT	CCATTGAAAT	GGAGTGCGGA	950
5	AGATCCCAAT	CTGTACACTT	TGGTTTTAAG	TTTAGTTGAT	CAGAATGGCA	1000
	AGTTGCTTGA	AACAGAAAGC	TGTCGCGTTG	GATTTCGTAA	ATTTGAACGC	1050
	AAGGACGGAT	TGATGCAAAT	TAATGGAAAG	CGGATTGTCT	TTAAAGGGAC	1100
10	AAATCGTCAC	GAATTCGCTT	CTGATAAAGG	TCGGGCGATA	ACGATAGATG	1150
	ATATGGTTAA	TGATATTCAG	CTGATGAAGC	AGCATAACAT	TAATGCCGTT	1200
15	CGAACCTCAC	ATTATCCGAA	TCATCCGCTT	TGGTATGAGT	TGTGTGATAC	1250
	GTATGGGTTA	TATGTGATTG	ACGAGACAAA	CTTAGAGACG	CACGGGACAT	1300
	GGGTTTATGG	TCAAAAAGGA	TTGGCTGAGA	CAATACCAGG	TAGTCTACCA	1350
20	AAGTGGACTG	AAAACGTCTT	GGATCGTTGT	AATTCAATGT	TCCAACGTGA	1400
	TAAAAACCAC	CCATCGATTC	TGGATTGGTC	ACTTGGTAAT	GAATCTTTTG	1450
25	GTGGCGATAA	CTTCTTGAAG	ATGCATGACT	TCTTTACGGA	ACAAGATCCA	1500
	GCTCGTCTGG	TGCACTATGA	GGGGATTTTT	CATTATCGTG	AATCTGAACG	1550
	GGCATCTGAT	ATGGAGAGTA	CCATGTATAT	TTCGCCAGAA	GGCATTGAGG	1600
30	ACTATGCAAA	GAAAGCGACC	AAGGAGACGA	AACCATATAT	TTTATGCGAA	1650
	TTCAGCCATG	CGATGGGCAA	CTCGCTAGGA	AACTTTTATA	AGTATACCGA	1700
35	GCTATTTGAT	CAATATCCGA	TCTTACAAGG	AGGCTTCATT	TGGGATTGGA	1750
	AGGATCAATC	GCTGCTAACG	AAGACAGCAG	GAGGCACACC	GTATCTTGCT	1800
	TATGGTGGTG	ATTTTGGTGA	ATCGCCACAC	GACGGCAACT	TTGCTGGTAA	1850
40	TGGTTTGATT	TTTGGAGATG	GCAAGGTTAG	CCCGAAGATT	TTTGAAGTGA	1900
	AGCGTTGTTA	CCAAAATGTT	GATTTCAAAG	CAATAGACTT	AGTGCACGGA	1950
45	CAAATCGAAT	TGACCAATAA	ATACTTGTTT	ACCAATCTCG	CTGACTACCA	2000
	ACTAAATTGG	GTTATCACTC	GAAACGGTGA	TGCAATAGAG	TCGGGTGCTA	2050
	CTAACATCAA	TGTCTTACCA	GGTGAAAAAA	GAGAGGTTAT	ACTTGACTAC	2100

ACGTTCCCAA CAGGCGTTTG CATGACGGAT GAATATATTT TGACCCTTCG 2150
TTTTTCTGAG AAAGGTGATC GCTTATGGTG TGAAGCGGGA CATGAAGTTG 2200
5 CATTTAATCA GTTTGTTTTA CCAACAAAAG TTACGAAATT ACGTGAGAAG 2250
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10 TACATCTGGT GCTGGATTTA GCGTCGGATT TGACACTAAA TCGGGTATGC 2350
TTGTTTCTTA CCAAGTTGGA GGTAATGAAT TGGTGAAAGA GGCACCTGTG 2400
CCAAACTTCT GGCGTGCAAT GACTGATAAT GATCGCGGGA ACGGACTCGA 2450
15 TCAACGGAGT CAGATTTGGC GTGATGCAAA TGAGGTACGT GAATTGGTTT 2500
CATTTCAGTA TGAAGTGTTG ACCAATAGAG TAAGCATATC AACGGTTTTTC 2550
20 TTATATGAAG ACCTCAACCA TTCACGCGTT GAACTTAACT TTTTGATTAC 2600
TGGAACCTGGT GAAATAAAGG TGGATTATGT ACTGAAACCG GGAGAAGATT 2650
TACCAGAAAT ACCAGAGATA GGTTTGATGT TAACGATGCC TAAGTCGTTT 2700
25 GATCAGTTAA GTTGGTATGG AAAAGGCCCA CATGAATCGT ATTGGGATAA 2750
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30 ATGTGCCGTA TTTGAAACCA CAAGAATGTG GCAACAAAGT AGGAGTTCGT 2850
TCAGCAGAAT TGGTTAATGA TGTGTTGTTT GGTTTGATTA TAAGTGGACT 2900
TCCAACGCTG GAGTTAAATG TCTTACCATA CACACCAGTG CAACTGGAAT 2950
35 CAGCTGATCA TAGCTATCAA TTACCAGAAA CAGATCAGAC TGTGTGCGT 3000
ATTAATTTAG GACAAATGGG AGTTGGTGGT GATGATAGTT GGGGACAGCG 3050
40 AACACACCAA GACTTTACCT TATTTGCAAA TAAAACCTAT CACTATAGCT 3100
TCATGTTGAA TAGTTTAAAC AGA 3123

SEQ ID NO: 3

(2) INFORMATION FOR SEQ ID NO: 3

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 nucleotides
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: synthetic DNA
- (xi) SEQUENCE DESCRIPTION SEQ ID NO: 3
- 15 CCGTCATCCA TATCACC

SEQ ID NO: 4

20 (2) INFORMATION FOR SEQ ID NO: 4

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 nucleotides
 (B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: synthetic DNA
- 30 (xi) SEQUENCE DESCRIPTION SEQ ID NO: 4
- CCTTTGCCCA AGAGCCAACC

35 **SEQ ID NO: 5**

(2) INFORMATION FOR SEQ ID NO: 5

- (i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 21 nucleotides
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 45 (ii) MOLECULE TYPE: synthetic DNA
- (xi) SEQUENCE DESCRIPTION SEQ ID NO: 5
- GCTATTATCA GACTTGGCAC C

SEQ ID NO: 6

5 (2) INFORMATION FOR SEQ ID NO: 6

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 21 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

15 (xi) SEQUENCE DESCRIPTION SEQ ID NO: 6

GTAATTCAAT GTTCCAACGT G

20 **SEQ ID NO: 7**

(2) INFORMATION FOR SEQ ID NO: 7

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 16 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION SEQ ID NO: 7

CGCTTATGGT GTGAAG

35

SEQ ID NO: 8

(2) INFORMATION FOR SEQ ID NO: 8

40

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION SEQ ID NO: 8

GGGCTGGCTT AACTATGCGG

5 **SEQ ID NO: 9**

(2) INFORMATION FOR SEQ ID NO: 9

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION SEQ ID NO: 9

20 CTGAATTCGC ATATGGCAA AAAATTAAAA AAATTC

SEQ ID NO: 10

(2) INFORMATION FOR SEQ ID NO: 10

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION SEQ ID NO: 10

35 CCAAGCTTAT CTGTTTAAAC TATTCAACAT G

SEQ ID NO: 11

40 (2) INFORMATION FOR SEQ ID NO: 11

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acid
45 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION SEQ ID NO: 11

GGCCATGGAT CCATGGCAAA AAAATTAAAA AAATTC

5

SEQ ID NO: 12

(2) INFORMATION FOR SEQ ID NO: 12

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION SEQ ID NO: 12

20 GGCCATCCCG GGTTATCTGT TTAAACTATT CAACATG

CLAIMS

1. A purified cold-active beta-galactosidase comprising the amino acid sequence as defined in SEQ ID NO. 1 or one comprising at least 90% identity to the full length amino acid sequence as defined in SEQ ID NO. 1, the amino acid sequence being selected so that the beta-galactosidase has a stable enzymatic activity at temperatures less than 8 °C and so that the beta-galactosidase exhibit the same biological activity as the amino acid sequence as defined in SEQ ID NO. 1.
2. The beta-galactosidase according to claim 1, wherein it is produced by a strain of an *Alkalilactibacillus*.
3. The beta-galactosidase according to claim 1 or 2, wherein it is produced by *Alkalilactibacillus ikkense*.
4. A nucleic acid molecule comprising a gene which encodes the beta-galactosidase according to any one of claims 1 to 3.
5. A nucleic acid molecule comprising SEQ ID NO. 2.
6. A recombinant vector comprising the nucleic acid molecule of claim 4 or 5.
7. The vector of claim 6, wherein said vector is an expression vector.
8. A host cell transformed with the vector of claim 6 or 7.
9. The cell according to claim 8, wherein the cell is selected from the group consisting of *Escherichia*, *Bacillus*,

Bifidobacterium, Lactococcus, Lactobacillus, Streptomyces, Leuconostoc, Streptomyces, Saccharomyces, Kluyveromyces, Candida, Torula, Torulopsis and Aspergillus.

10. The cell of claim 9, wherein the cell is selected from the group consisting of *Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium bifidum, Bifidobacterium animalis*, and *Lactococcus lactis*.
11. Use of the cell of any one of claims 8 to 10 for producing a product selected from the group consisting of lactose-free milk, low-lactose milk, yoghurt, cheese, fermented milk products, dietary supplement and probiotic comestible product.
12. Use according to claim 11 for producing a dairy product with a lactose concentration at 1% or lower.
13. Use according to claim 12, wherein the lactose concentration is 0.1% or lower.
14. Use according to claim 13, wherein the lactose concentration is 0.01% or lower.
15. Use of the beta-galactosidase according to any one of claims 1 to 3 for producing a product selected from the group consisting of lactose-free milk, low-lactose milk, yoghurt, cheese, fermented milk products, dietary supplement and probiotic comestible product.
16. Use according to claim 15 for producing a dairy product with a lactose concentration at 1% or lower.

17. Use according to claim 16, wherein the lactose concentration is 0.1% or lower.
18. Use according to claim 17, wherein the lactose concentration is 0.01% or lower.
19. A process for producing the beta-galactosidase of any one of claims 1 to 3, comprising culturing the cell of any one of claims 8 to 10 in a suitable culture medium under conditions permitting expression of said beta-galactosidase, and recovering the resulting beta-galactosidase from the culture.
20. The process according to claim 19, wherein the resulting beta-galactosidase is immobilized.
21. A process for hydrolysis of lactose, comprising contacting of the beta-galactosidase of any one of the claims 1 to 3 or the cell of any one of the claims 8 to 10 with a solution of lactose.
22. A psychrophilic bacterium *Alkalilactibacillus ikkense* BCCM Accession No LMG P-24866.

Figure 1

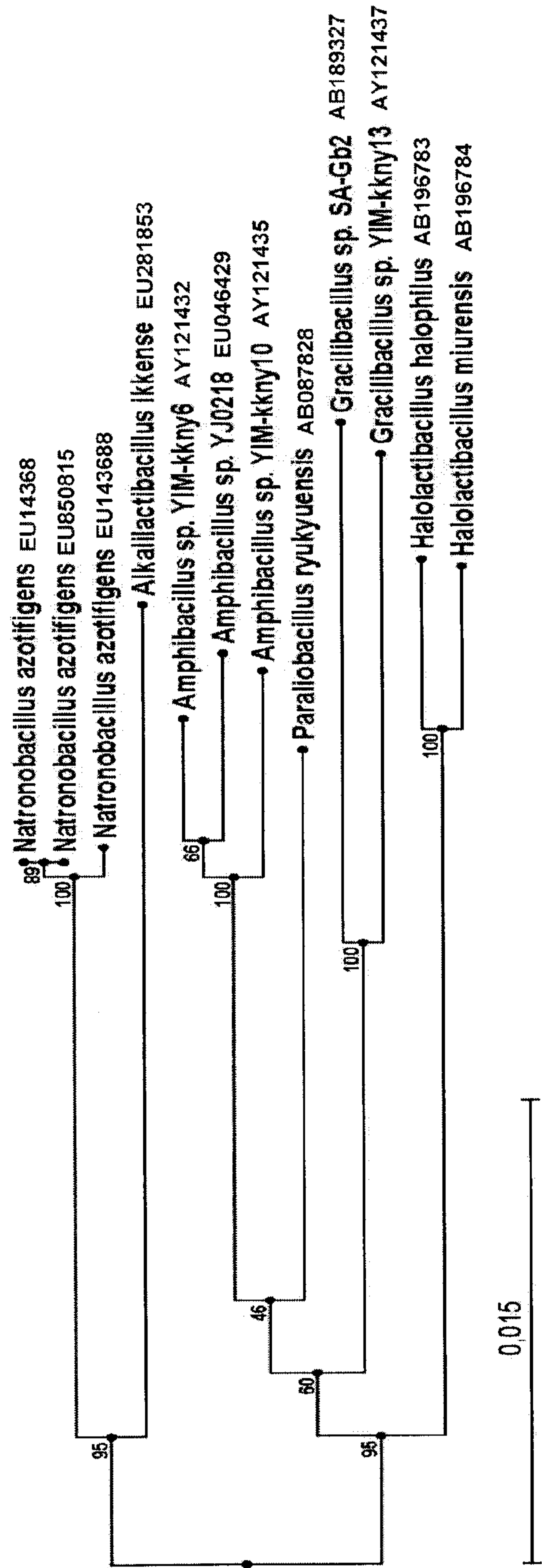


Figure 2

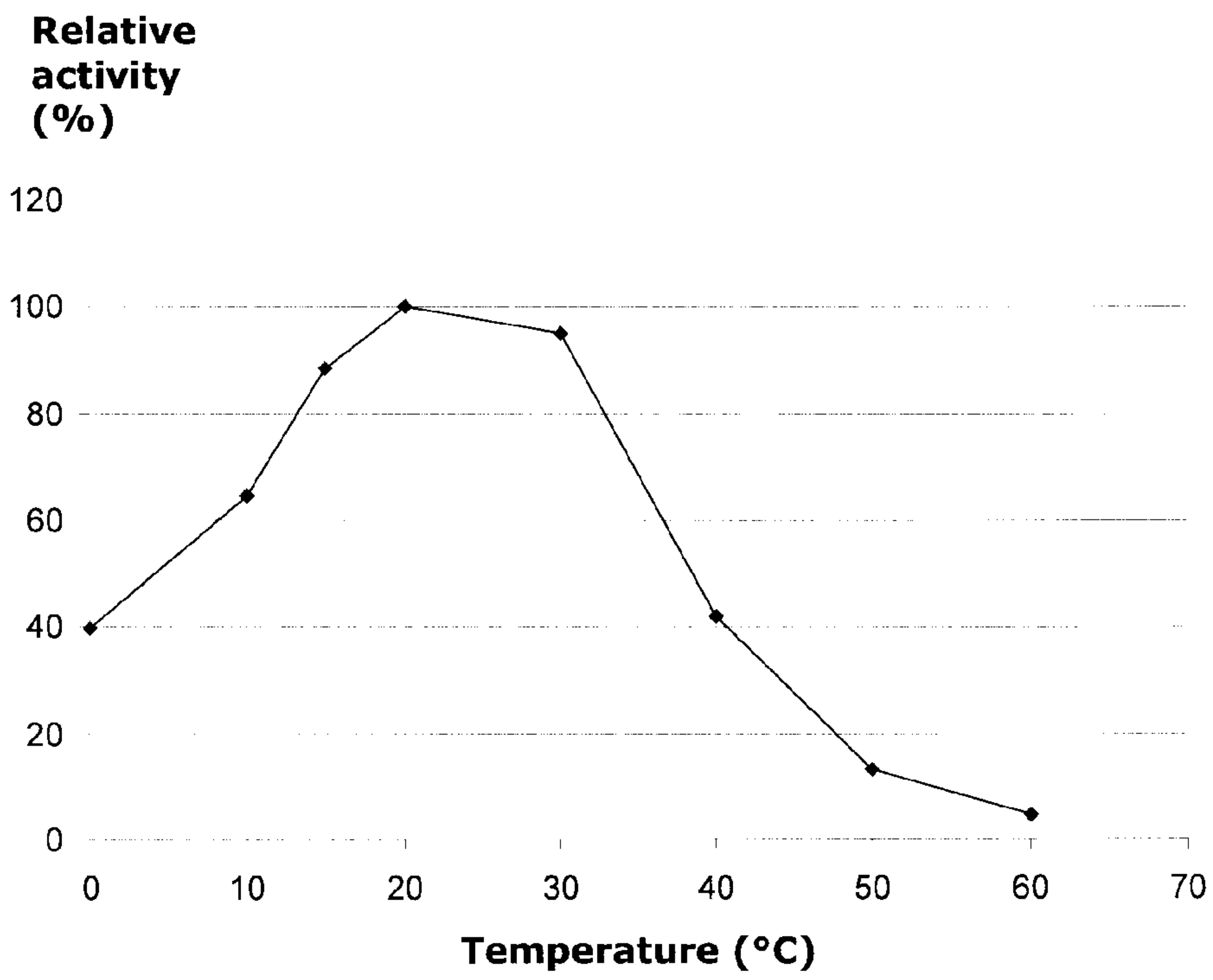


Figure 3

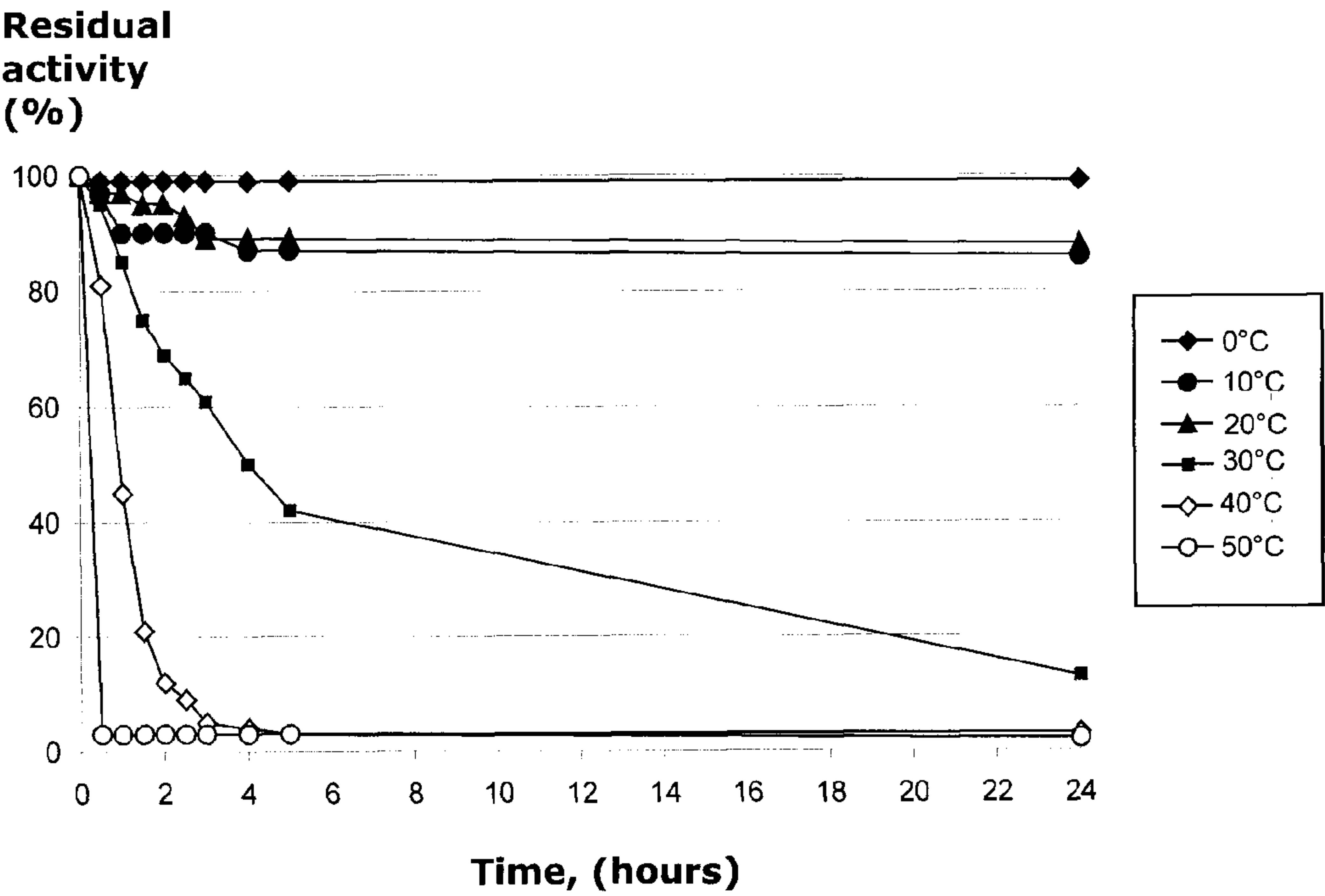


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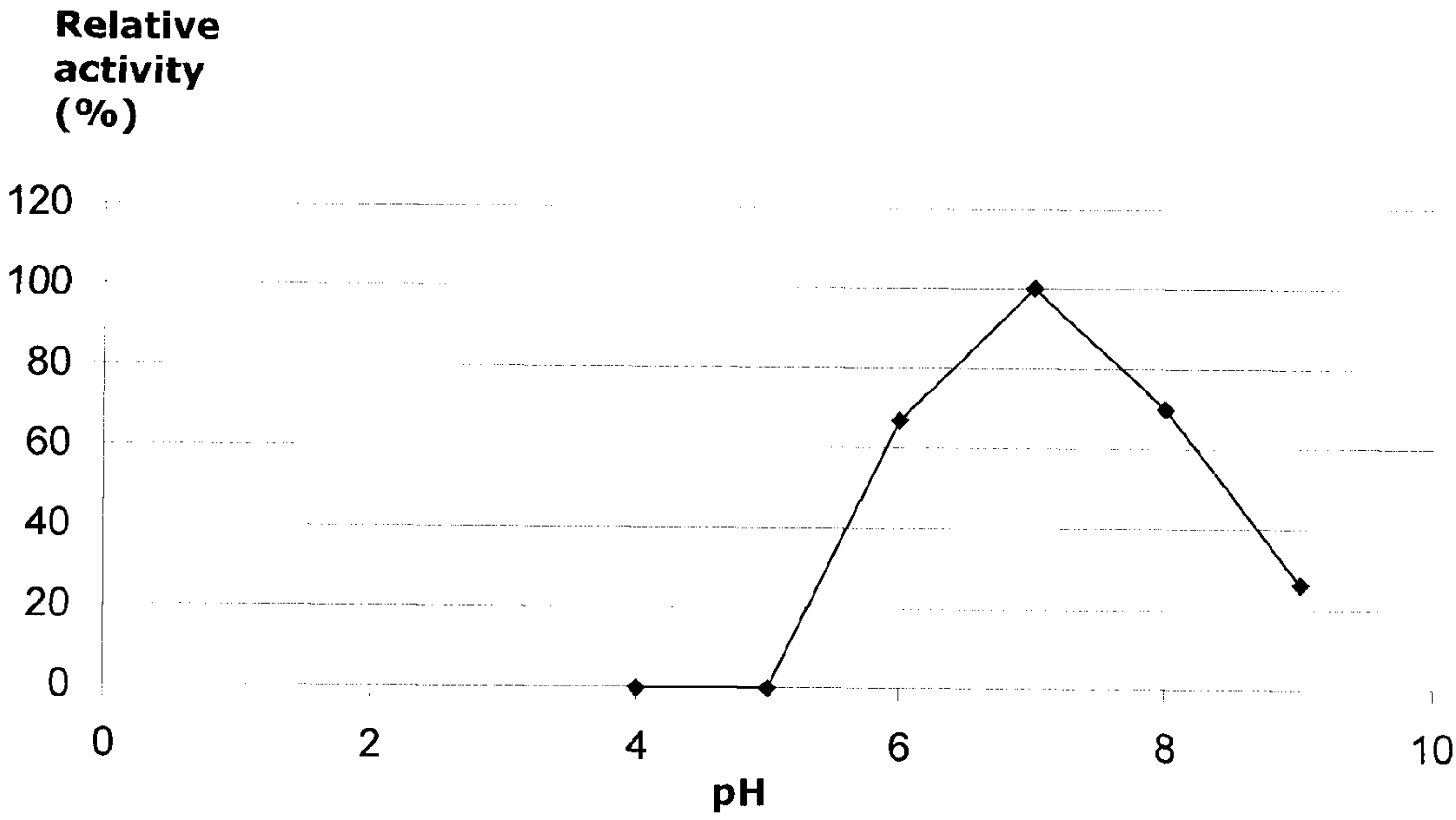


Figure 5

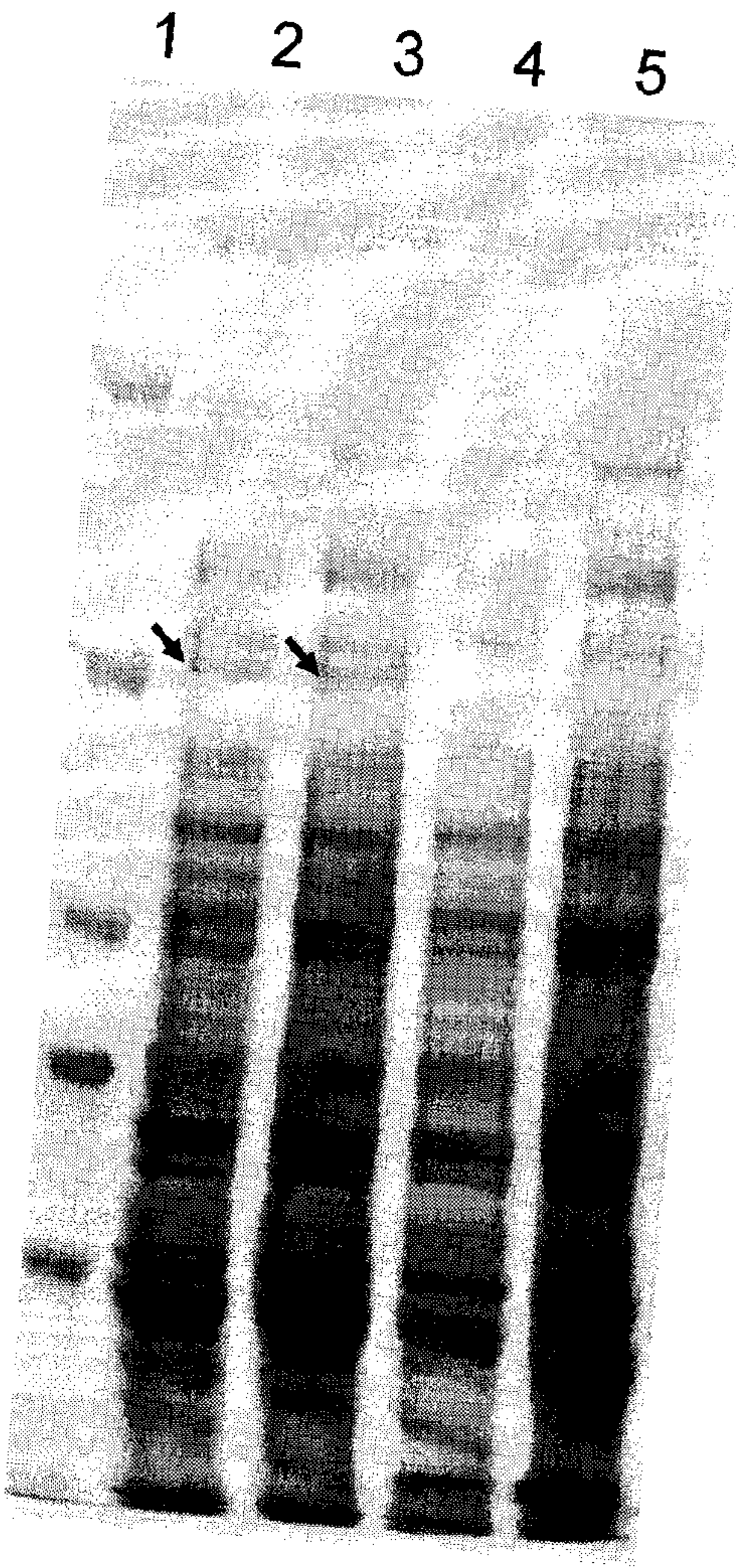


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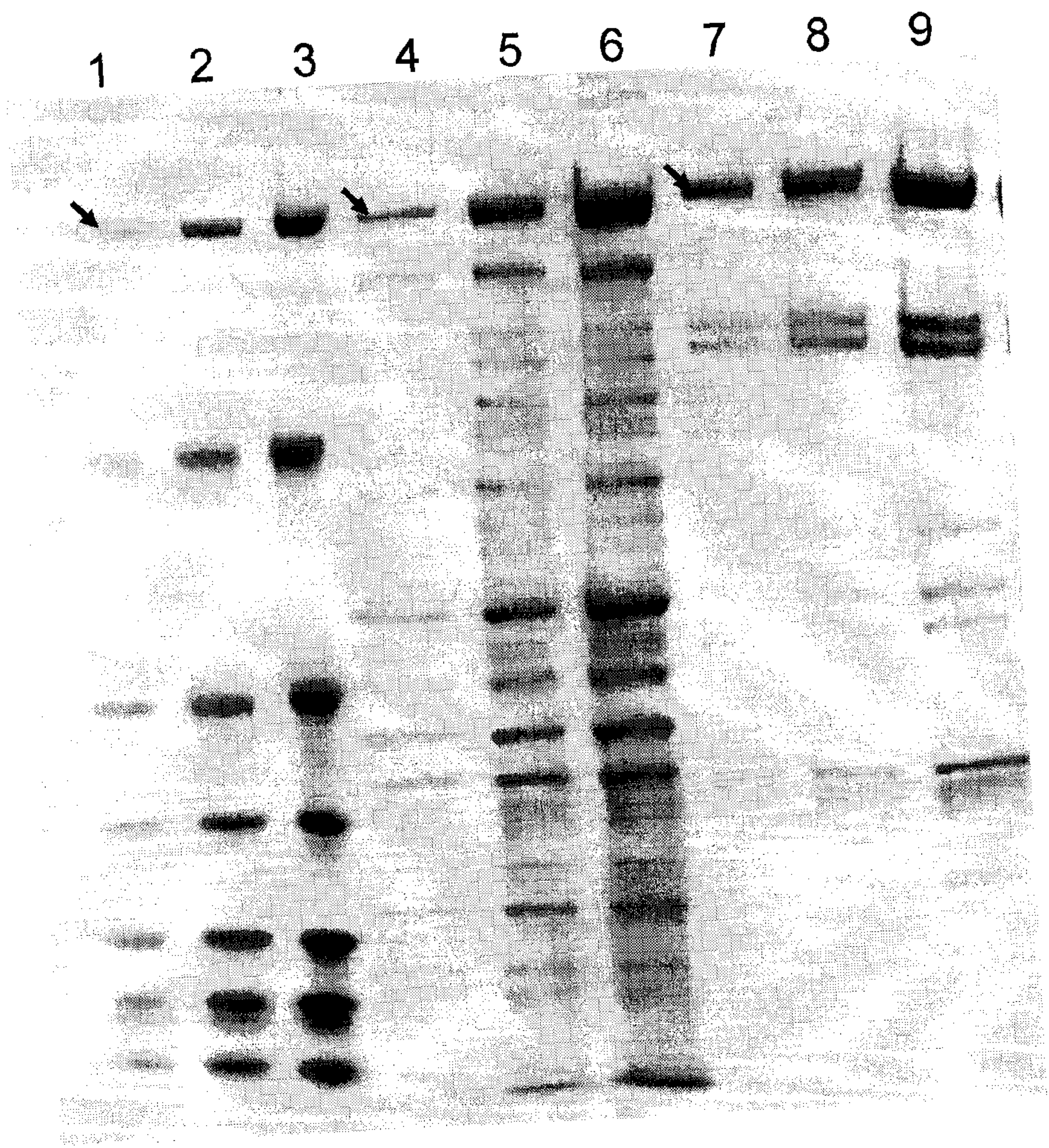


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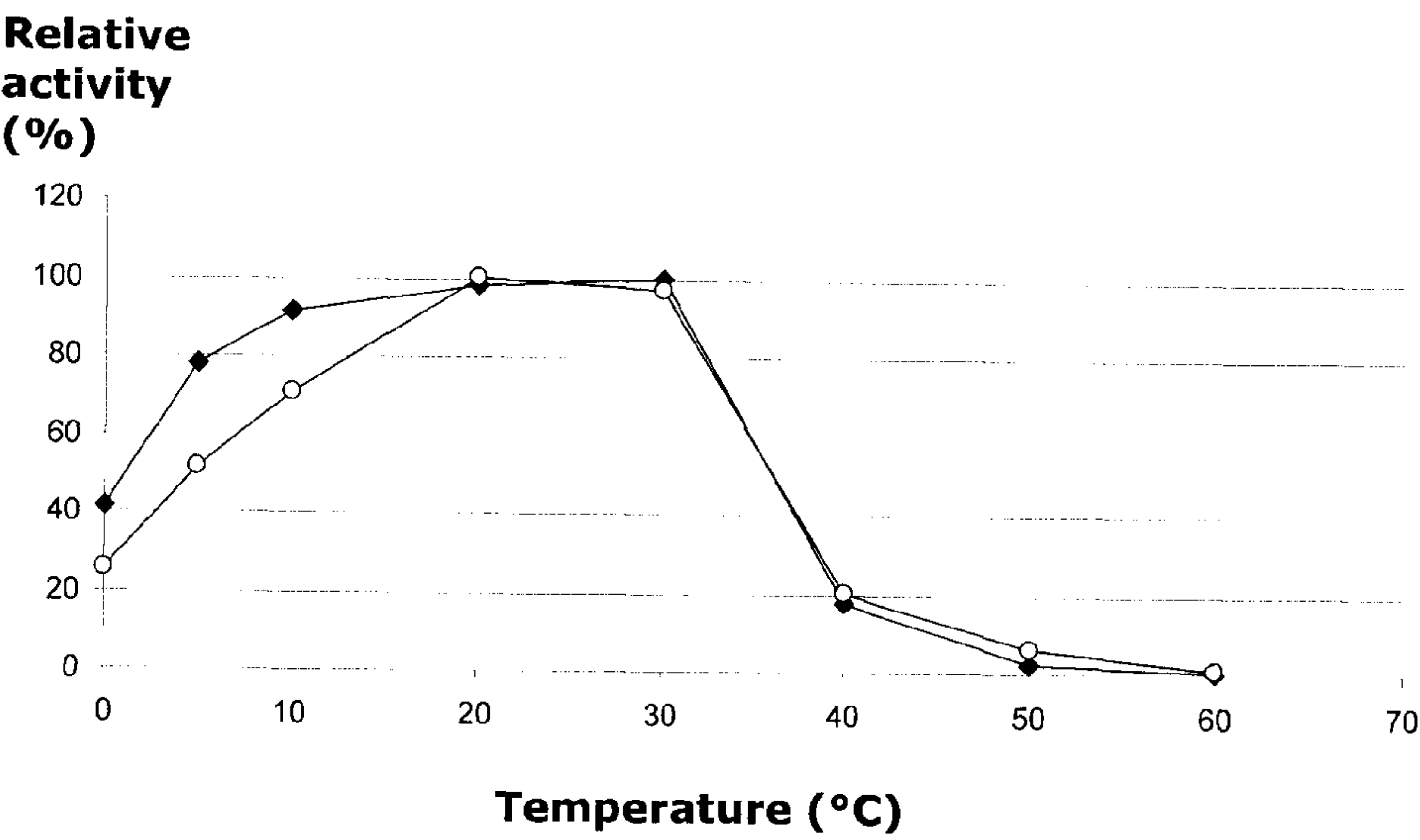


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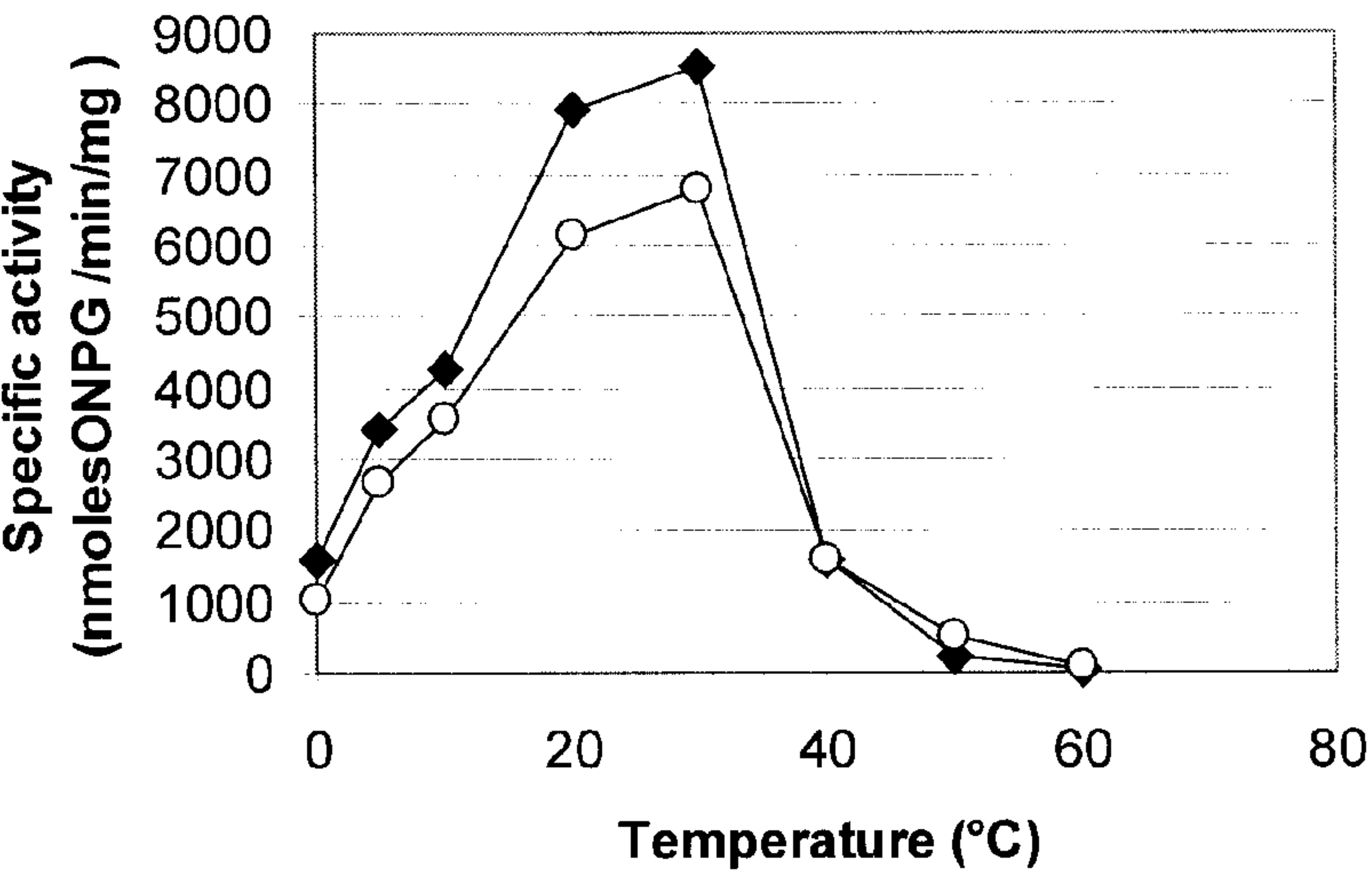


Figure 9

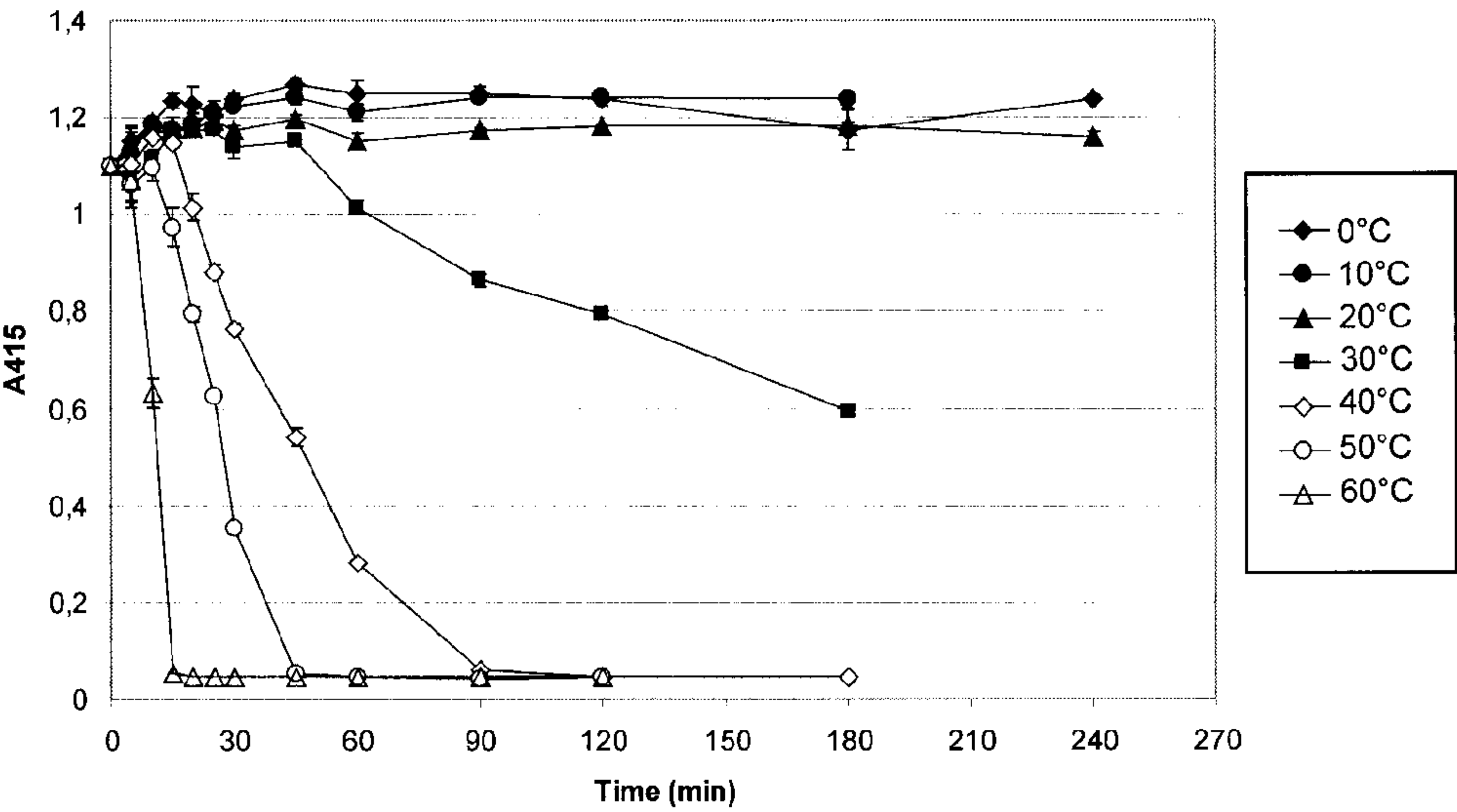


Figure 10

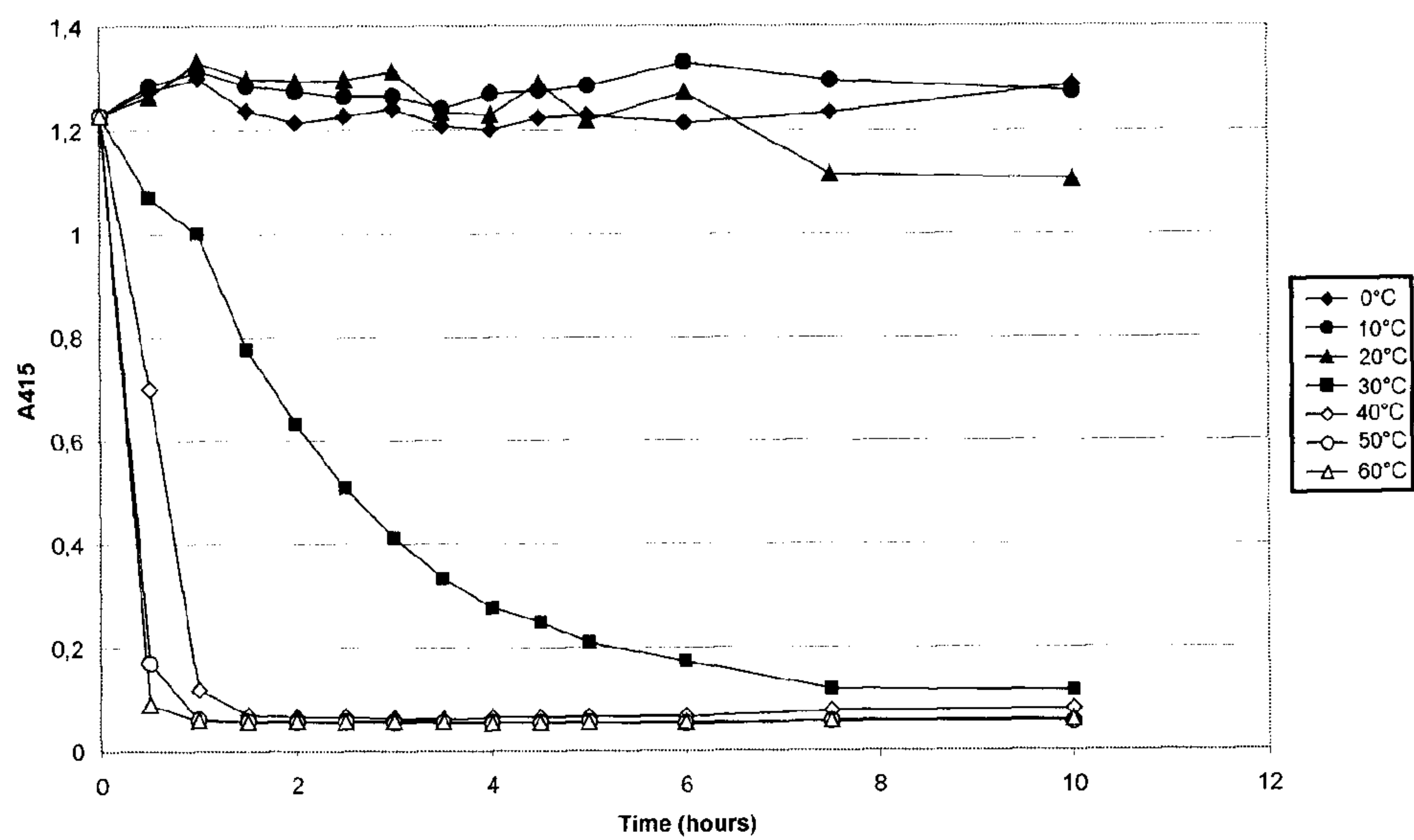


Figure 11

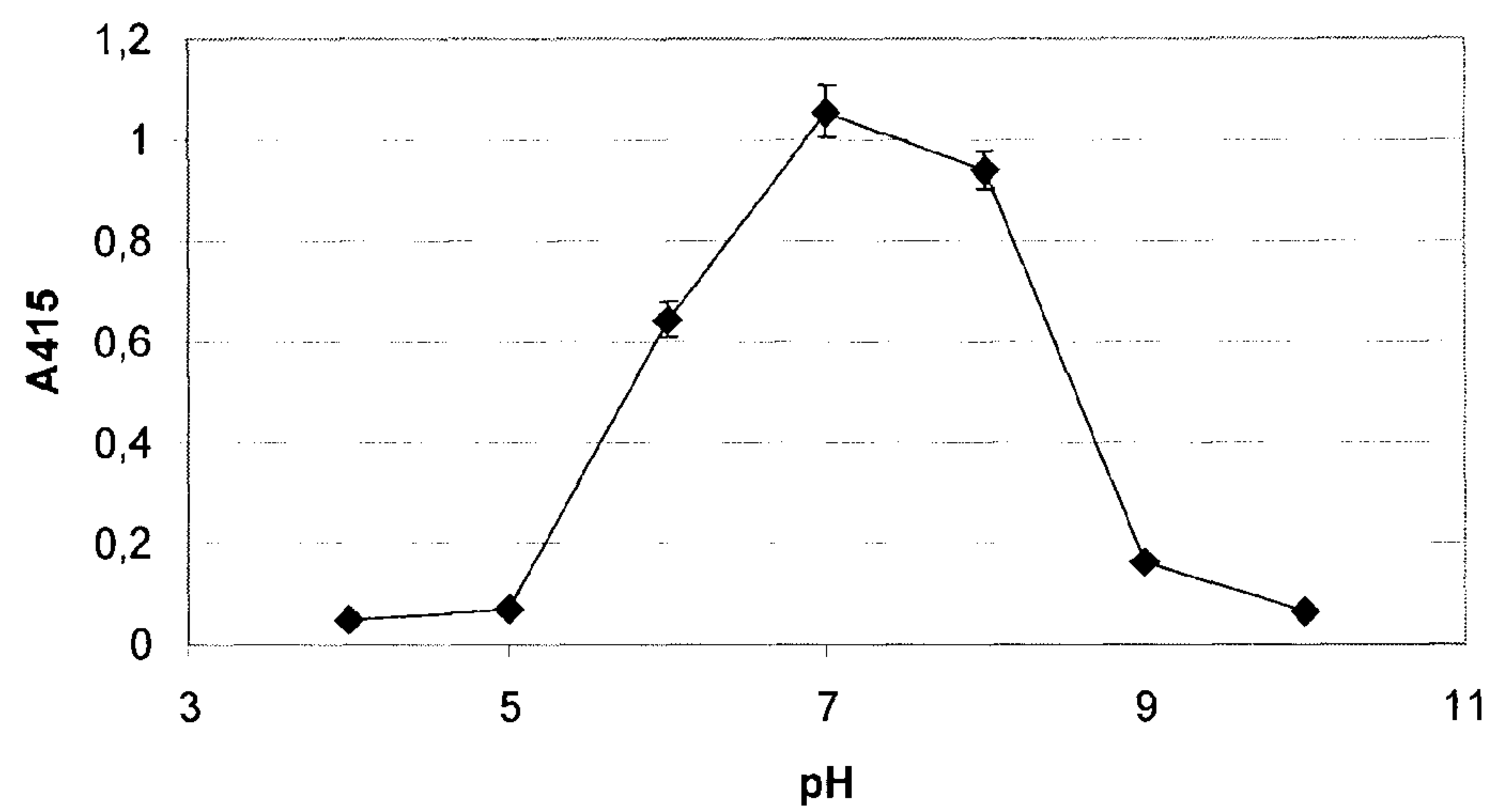


Figure 12A

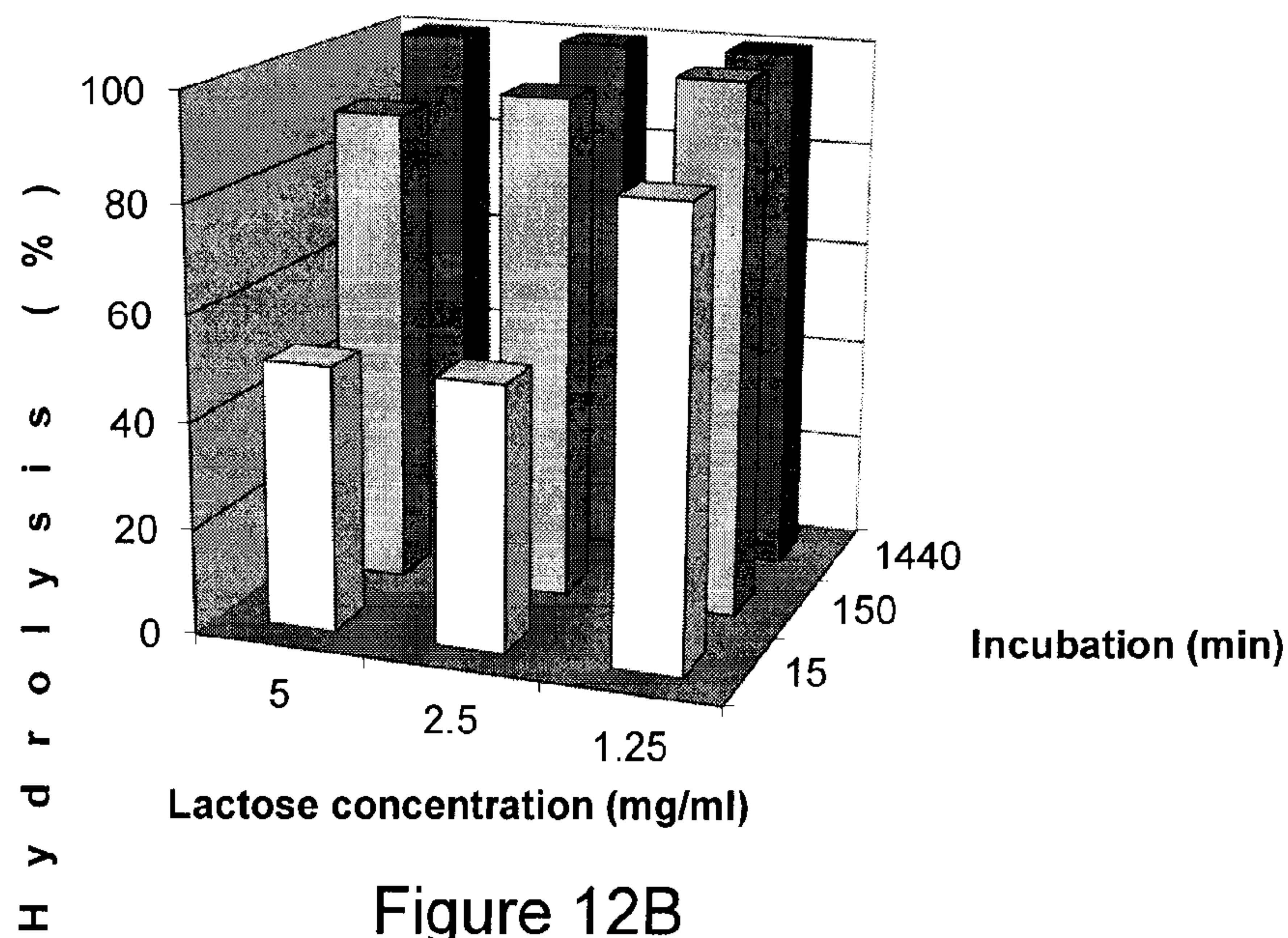
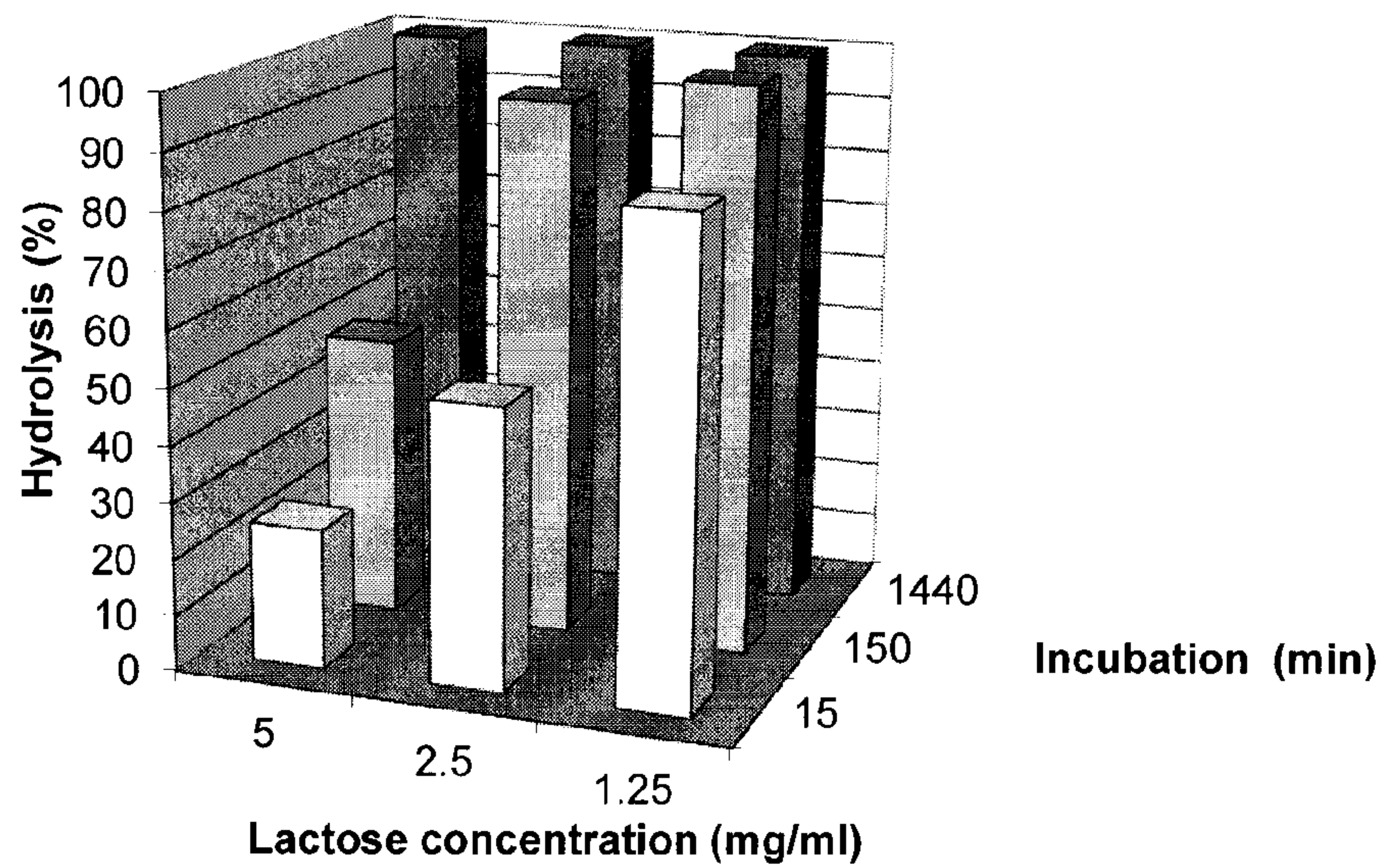


Figure 12B

Figure 13

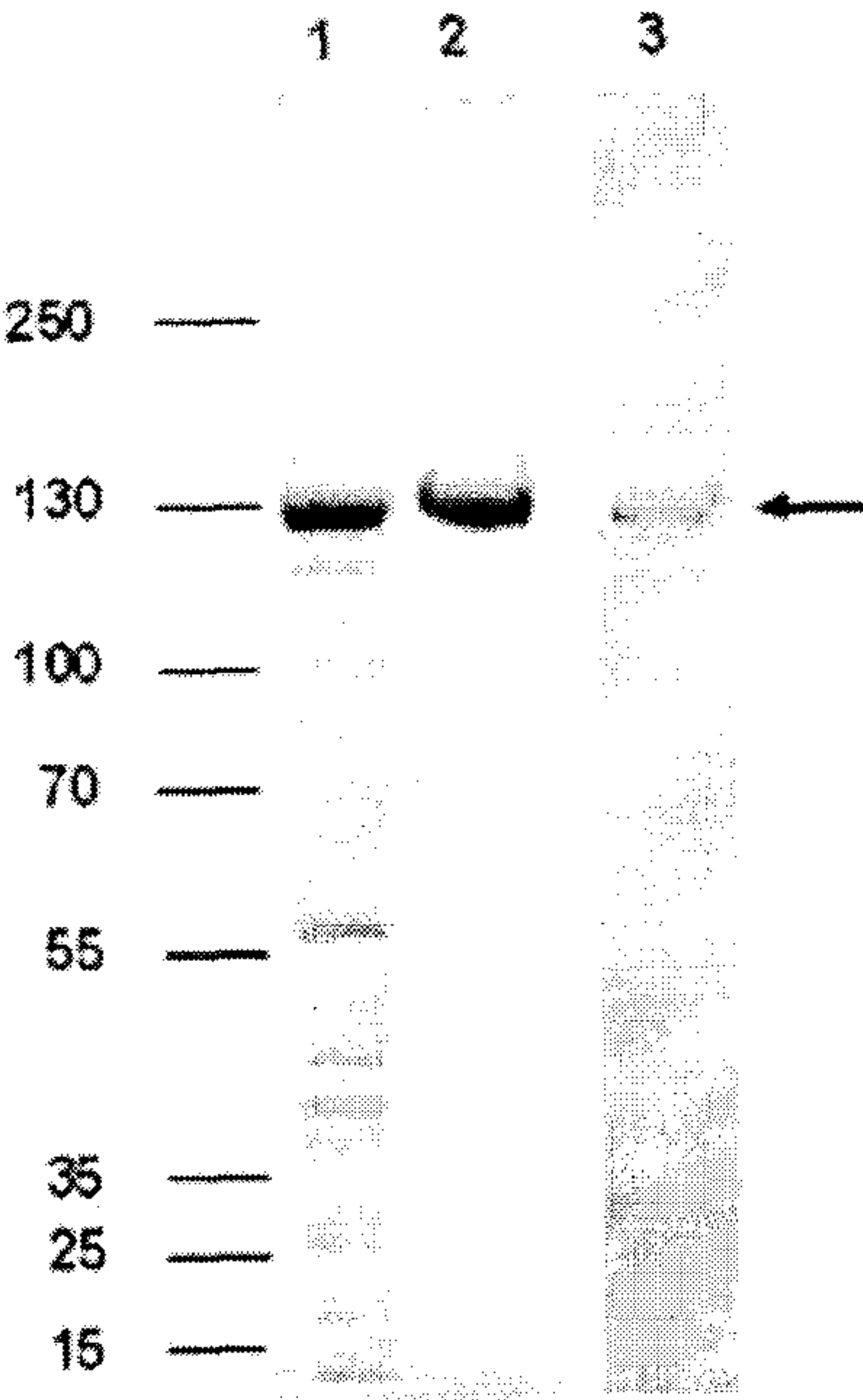


Figure 14

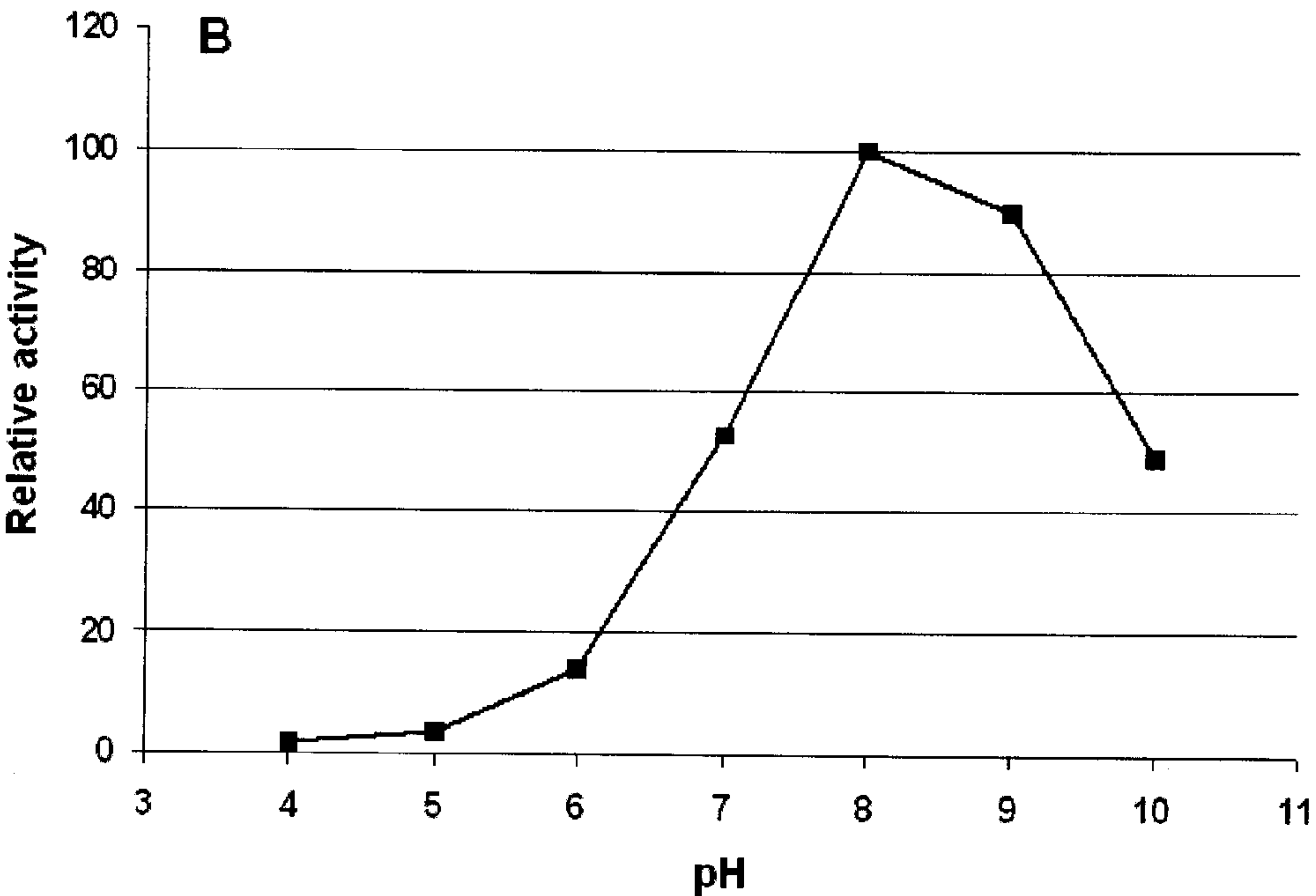
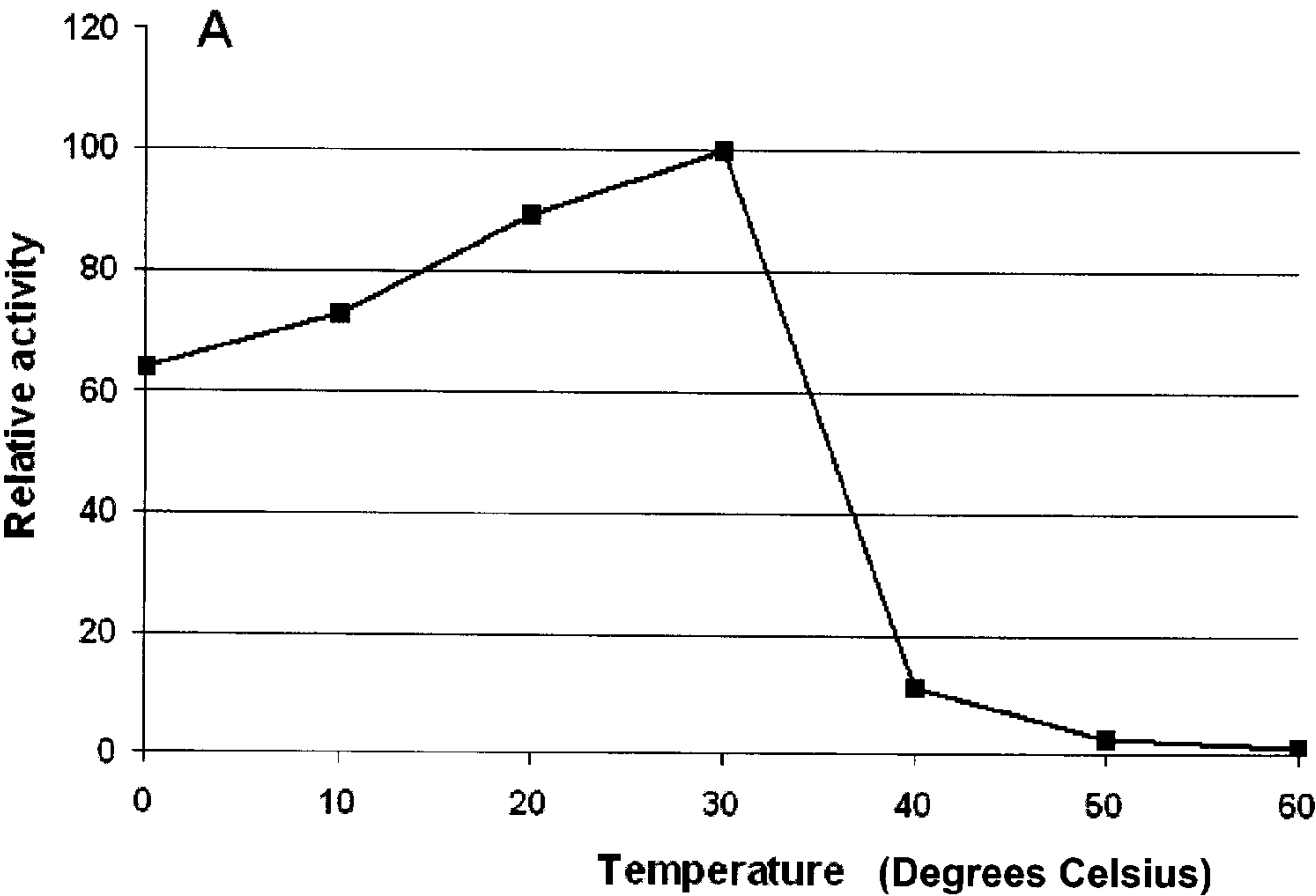


Figure 15

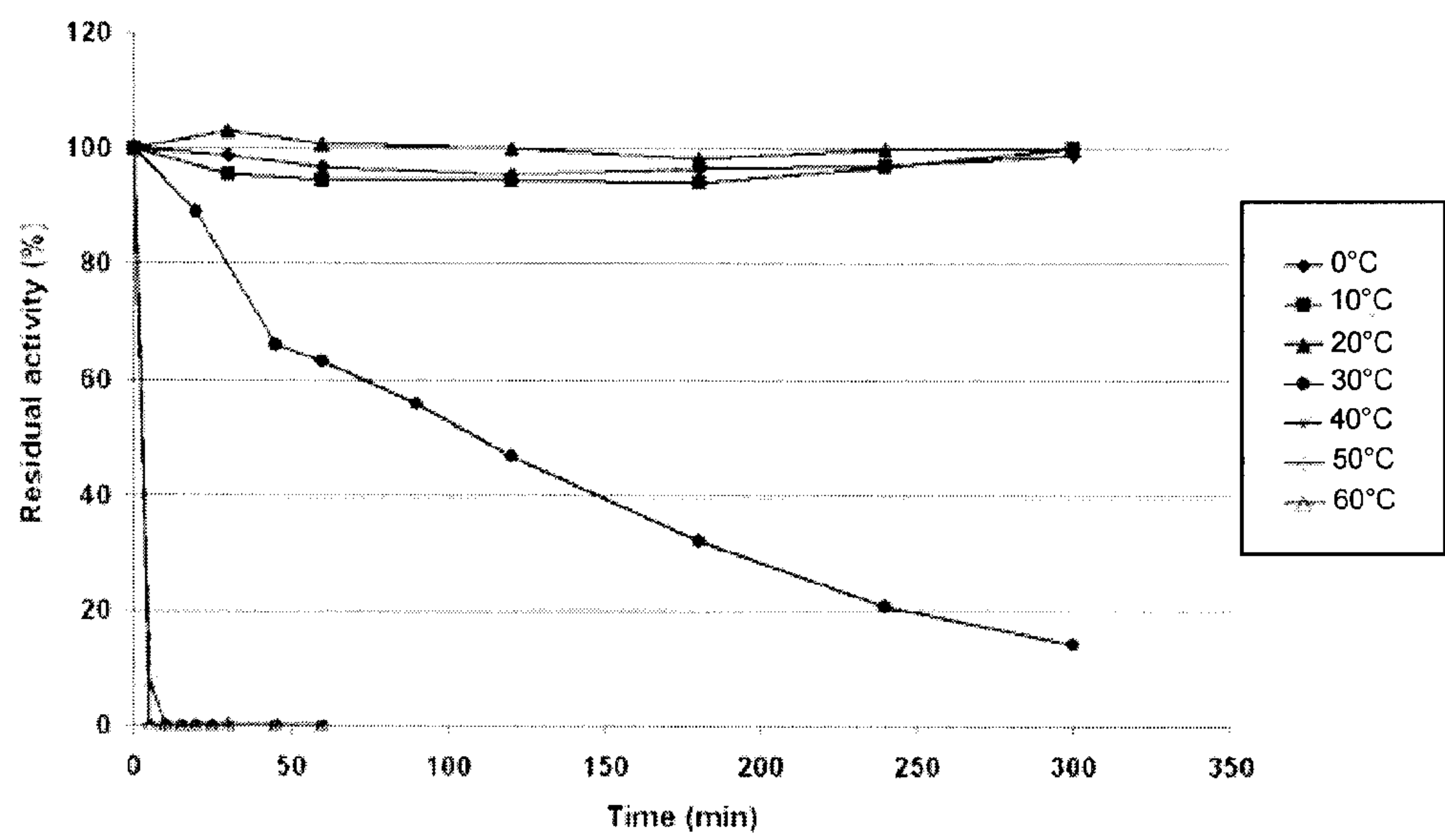


Figure 16

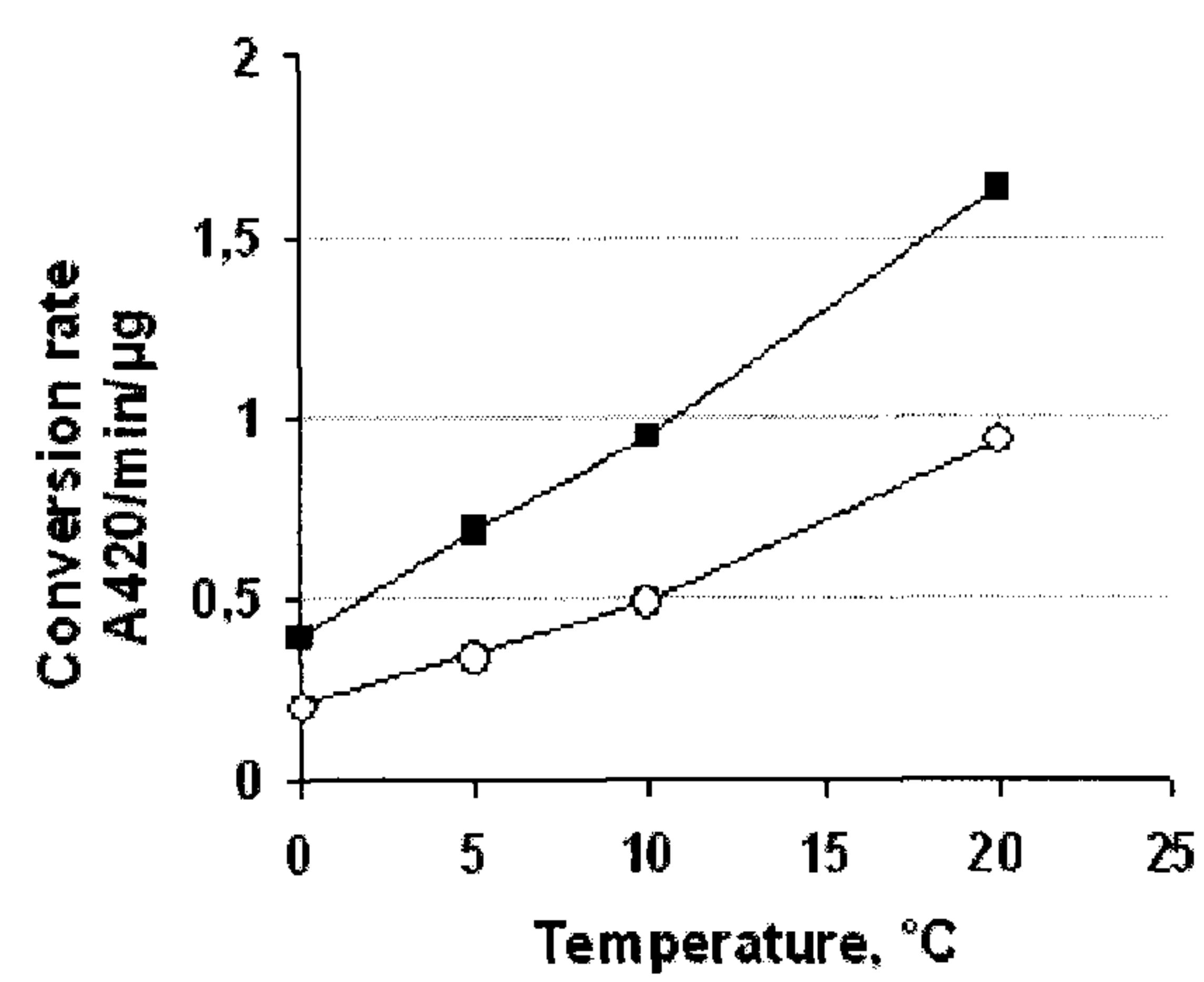


Figure 17

