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APPENDIX 6

Xylanase from *Bacillus licheniformis*

An application to amend the *Australia New Zealand Food Standards Code* with a xylanase preparation produced by a genetically modified *Bacillus licheniformis*

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Appendix 6

Non-CCI version

Elements in Appendix 6 that are to be treated as confidential commercial information (CCI) are marked in highlighted text in the CCI version and the corresponding text as [redacted] in the non-CCI version.

Documentation regarding the production strain

1. Detailed description of the construction of the genetically modified production strain
2. Homology assessment of the GH8 xylanase concluding that the *Bacillus sp. KK-1* belong to the species *Bacillus licheniformis*
3. DNA sequence of the *xyl264* gene and the amino acid sequence of the xylanase264 variant
4. Evidence of natural occurrence of [redacted]
5. Introduced DNA sequences in the production strain
6. Genetic stability of the production strain (Southern blot). Novozymes Report No.: 2012-12765

Appendix 6.1

Detailed description of the construction of the genetically modified production strain

6.1.1. The host organism

Taxonomy

The host strain, designated *B. licheniformis* BW302, was derived from strain Si3, a sporulation-deficient derivate of a natural isolate of *B. licheniformis*, ATCC 9789 (named Ca63). The taxonomic classification is as follows:

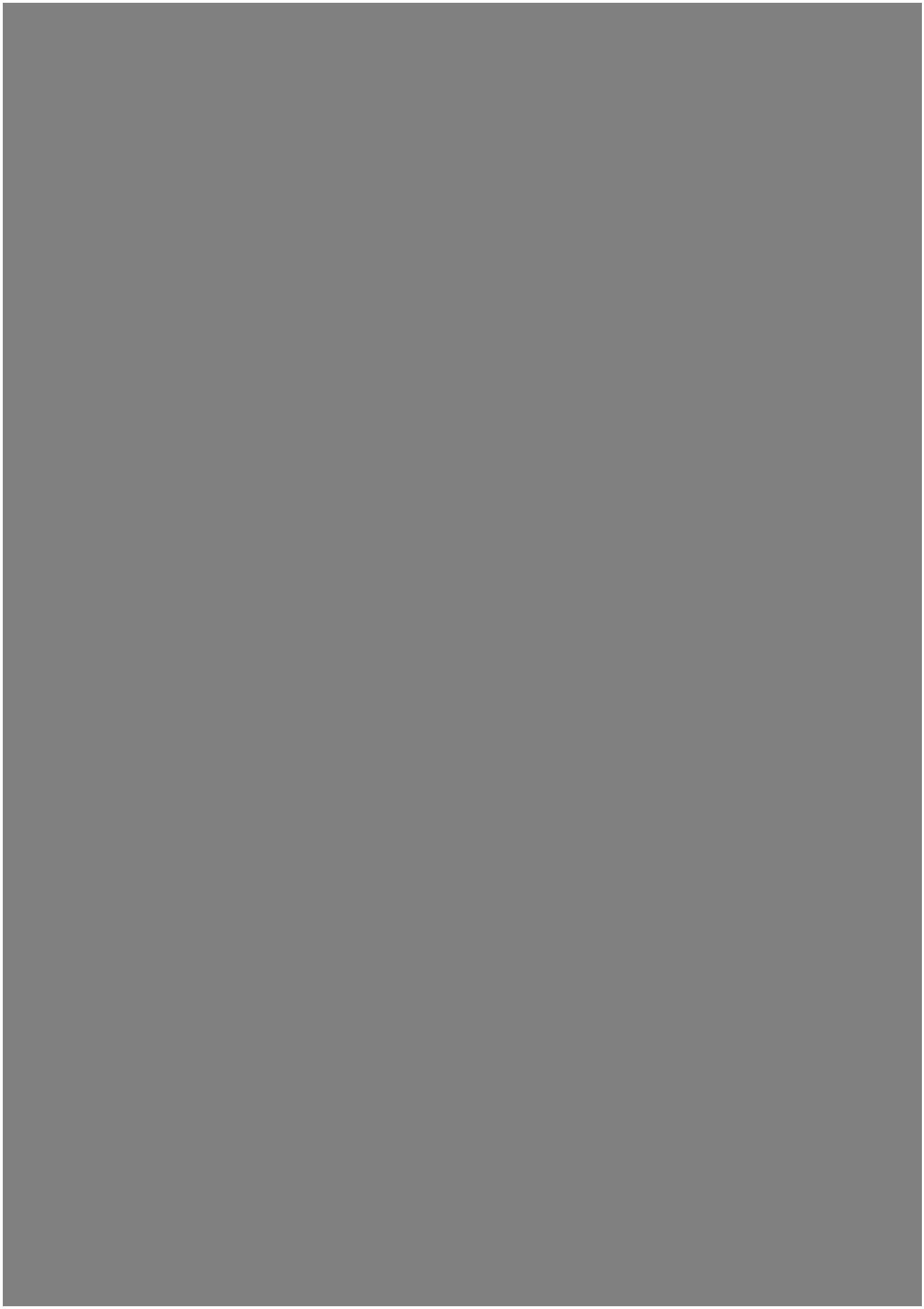
Name:	*Bacillus licheniformis*
Class:	Bacilli
Order:	*Bacillales*
Genus	*Bacillus*
Species:	*licheniformis*

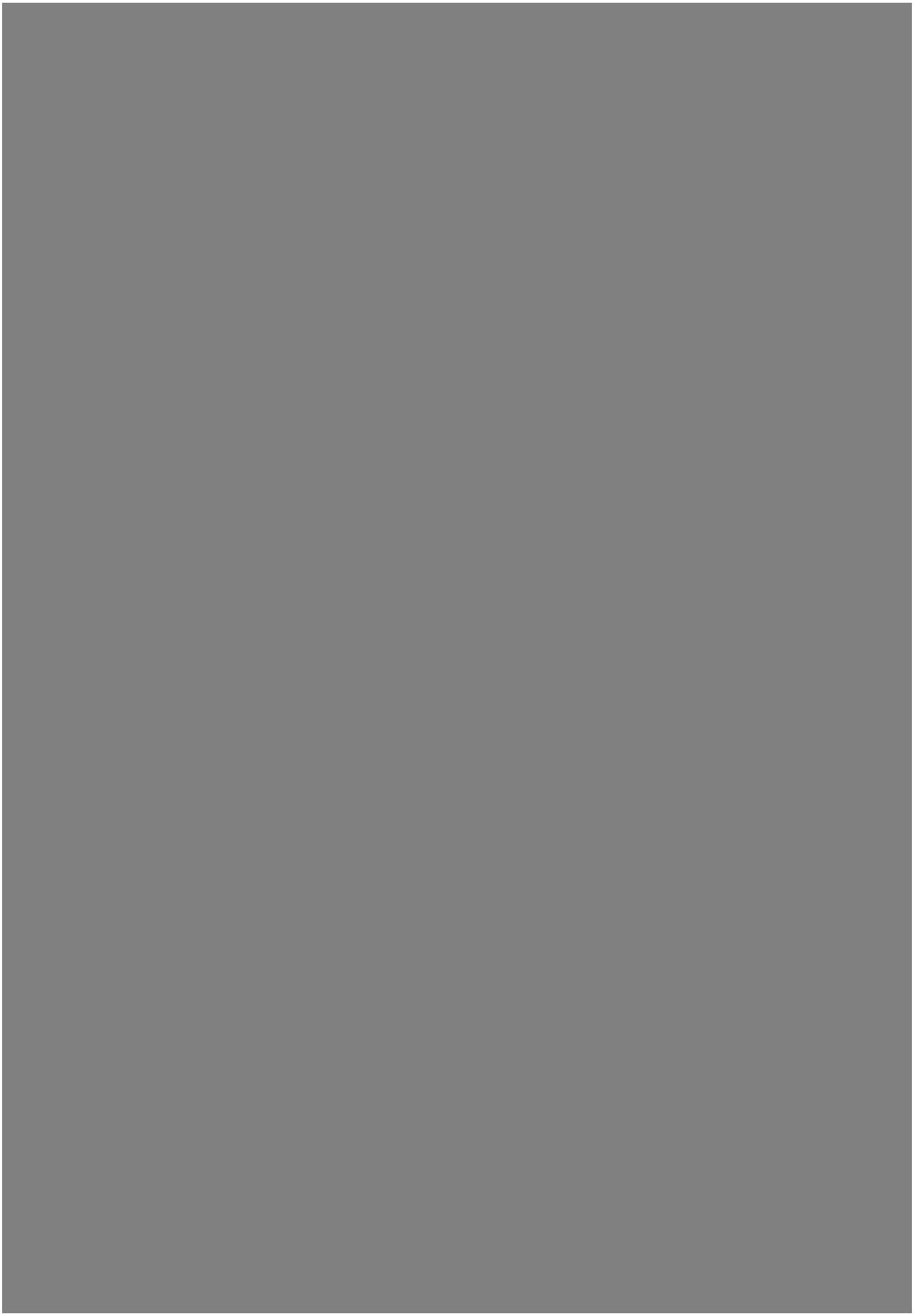
The classification of the natural isolate was confirmed by Deutsche Sammlung von Mikrooorganismen und Zellkulturen GmbH.

Genetic modifications

The host strain, BW302, was modified at several chromosomal loci to cause deletion of the [redacted]. Also, [redacted] eliminating the ability to sporulate, as well as genes for unwanted peptides, [redacted] was deleted.

[redacted]





[redacted]

6.1.2. Origin and donor of vector and inserts

The enzyme gene

The product gene, designated *xyl264*, is not isolated from a donor strain, but fully synthesized based on sequence data from a public database [redacted]. According to Yoon *et al*, 1998[4], the gene is derived from a thermophilic soil bacterium *Bacillus sp. KK-1*. The unmodified GH8 xylanase encoded by this gene is referred to as a thermostable, cellulase-free, xylanase (Kim *et al*, 1995[5]).

Based on homology assessment of the GH8 xylanase to public databases showing 98.5% homology to another xylanase from *Bacillus licheniformis*, it is concluded that the strain *Bacillus sp. KK-1* belongs to the same species, *Bacillus licheniformis*. The report is enclosed as Appendix 6.2.

The xylanase264 variant is a protein engineered variant of the GH8 xylanase, [redacted]. The xylanase264 variant and the wildtype GH8 xylanase otherwise show 100% aligned sequence identity excluding the aligned gap introduced at the insertion position.

The DNA sequence of the *xyl264* gene and the amino acid sequence of the xylanase264 variant are shown in Appendix 6.3.

Wildtype GH8 xylanases from strains within the *Bacillus licheniformis* species show as low as 91.5% aligned sequence identity over their mature amino-acid sequences. A single residue [redacted] in xylanase264 is comparable to a 1 in 400 residue change, or 99.75% sequence identity. Therefore, the change resulting from the protein engineering is insignificant compared to the interspecies variation found in nature.

Furthermore, there is indeed evidence that the specific change [redacted] described above occurs in nature. [redacted]

Promoter

[redacted]

Terminator

The *amyL* terminator originates from parental strain *B. licheniformis* Ca63.

Vector/insert

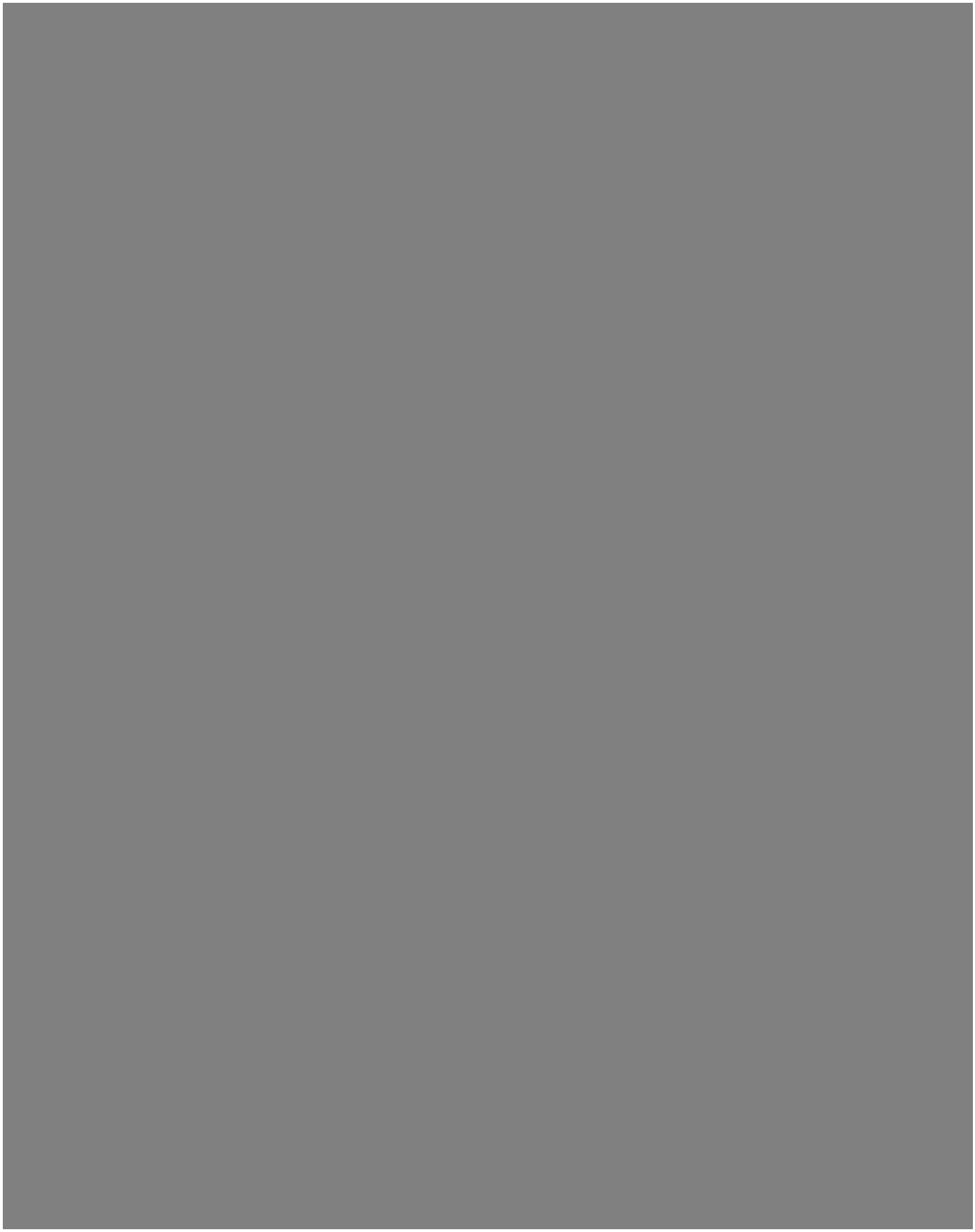
Vectors used are composed of elements from plasmids [redacted] pE194 (Horinouchi, S. and Weisblum, B., 1982[8]) [redacted].

6.1.3. Introduced genetic sequence

[redacted]

Diagrams of the xylanase264 expression cassette, which is integrated into [redacted] the *B. licheniformis* chromosome and tables outlining the introduced genetic elements, as well as the full sequence of the DNA that was introduced as a result of the genetic modifications is given in Appendix 6.3.

6.1.4. Construction of the recombinant production organism



The production strain, *B. licheniformis* HyGe329, was constructed from BW302 through the following steps:

1) A conjugation donor strain harbouring pBW120 was used to mobilize pBW120 into the recipient strain BW302,

2) pBW120 was integrated into strain BW302

3)

4) The resulting two-copy xylanase264 strain was named HyGe329.

The DNA segments inserted into the chromosome of the final production strain comprise the sequence of the *xyl264* gene, encoding the xylanase264 variant, . The gene is transcribed using the promoter and the *amyL* terminator described in section 2.4.2.

6.1.5. Description of the production organism

The chromosome of the final production strain HyGe329 has been modified at different positions relative to the non-recombinant strain Ca63. The modifications have led to the following changes in the strain:

- Deletions have been introduced in the [redacted] genes. The strain is thus unable to produce these [redacted] proteases.
- [redacted]
- A deletion has been introduced in [redacted]. The strain is thus unable to sporulate.
- [redacted]

Identity and taxonomy of production organism

The production strain is a *Bacillus licheniformis* carrying two genes coding for a xylanase variant from *Bacillus licheniformis*.

Genetic stability and mobilization and conjugation capability

The inserted recombinant DNA is genetically stable during fermentation, as the inserted DNA is integrated into the chromosome.

The genetic stability of the production strain was tested at large-scale fermentation. The strain stability during fermentation was analyzed by Southern blotting. No instability of the strain was observed (Appendix 6.4).

As the insert is chromosomally integrated and lacks a functional origin of replication, it cannot be transferred by conjugation to other organisms, nor can fragments replicate autonomously.

Antibiotic resistance gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications.

Appendix 6.2

Homology assessment of the GH8 xylanase concluding that the *Bacillus sp. KK-1* belong to the species *Bacillus licheniformis*

The gene encoding the GH8 xylanase is not isolated from a donor strain, but fully synthesized based on sequence data from a public database [redacted]. GH8 xylanase is derived from *Bacillus sp. KK-1*.

In public databases a nearly identical xylanase exists [redacted]. The GH8 xylanase from [redacted] and [redacted] are 98.5% identical. [redacted] is isolated from a bacteria that based on 16S analysis is classified as a *Bacillus licheniformis* [redacted].

It has been documented that there is a very good correlation between the 16S sequence identity between two strains and the amino acid identity of proteins between the two strains (Konstantinidis K.T., and Tiedje J.M., 2007[11]). This means that two proteins with high amino acid identity originate from strains that have 16S genes that are nearly identical. The existence of proteins with more than 95% sequence identity correlates with 16S sequence identity above 99%.

Strains with 16S sequence identity above 99% are considered to be from the same species (Stackebrandt E. and Goebel B.M., 1994[12]).

In accordance with above it is concluded that the high sequence identity of the two genes (the GH8 xylanase from *Bacillus sp. KK-1* [redacted] and [redacted] (see below) shows that the origin of the two genes is within the same species: *Bacillus licheniformis*.

[redacted]

Appendix 6.3

DNA sequence of the *xyl264* gene and the amino acid sequence of the xylanase264 variant

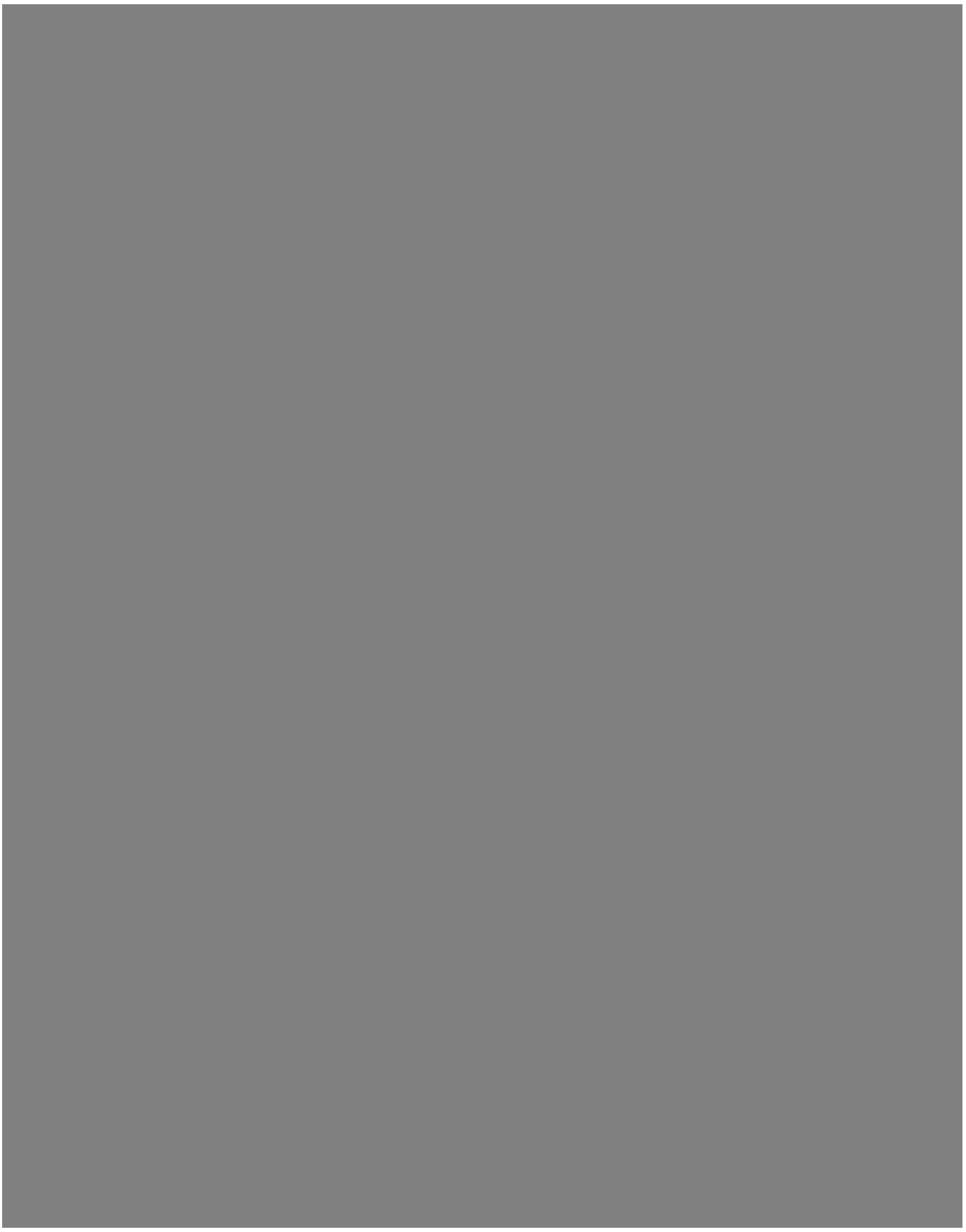
The DNA sequence of the *xyl264* gene is the following:

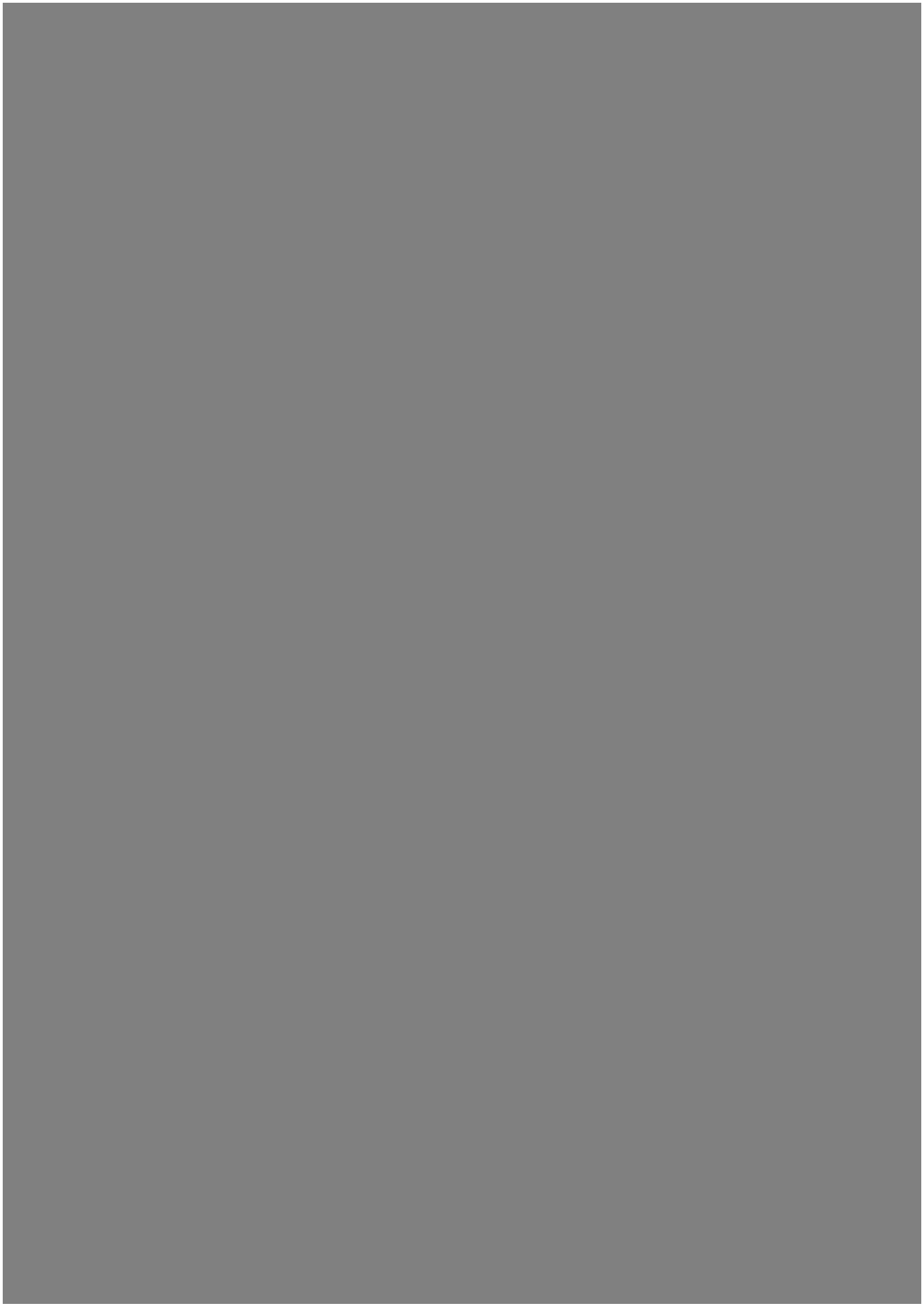
The amino acid sequence of the xylanase264 deduced from the DNA sequence shown above is:

Appendix 6.4

Evidence of natural occurrence [redacted]

[redacted]



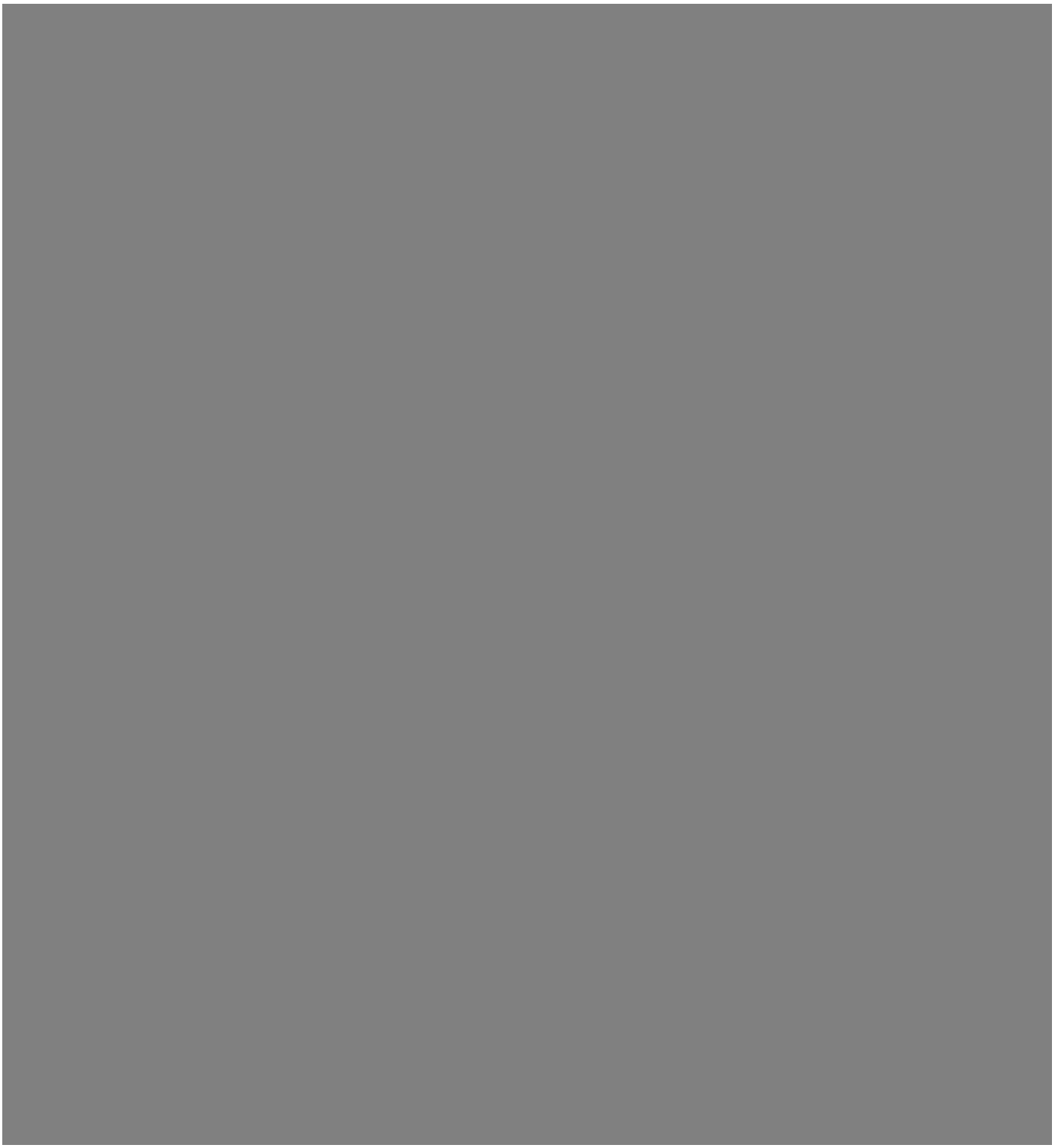


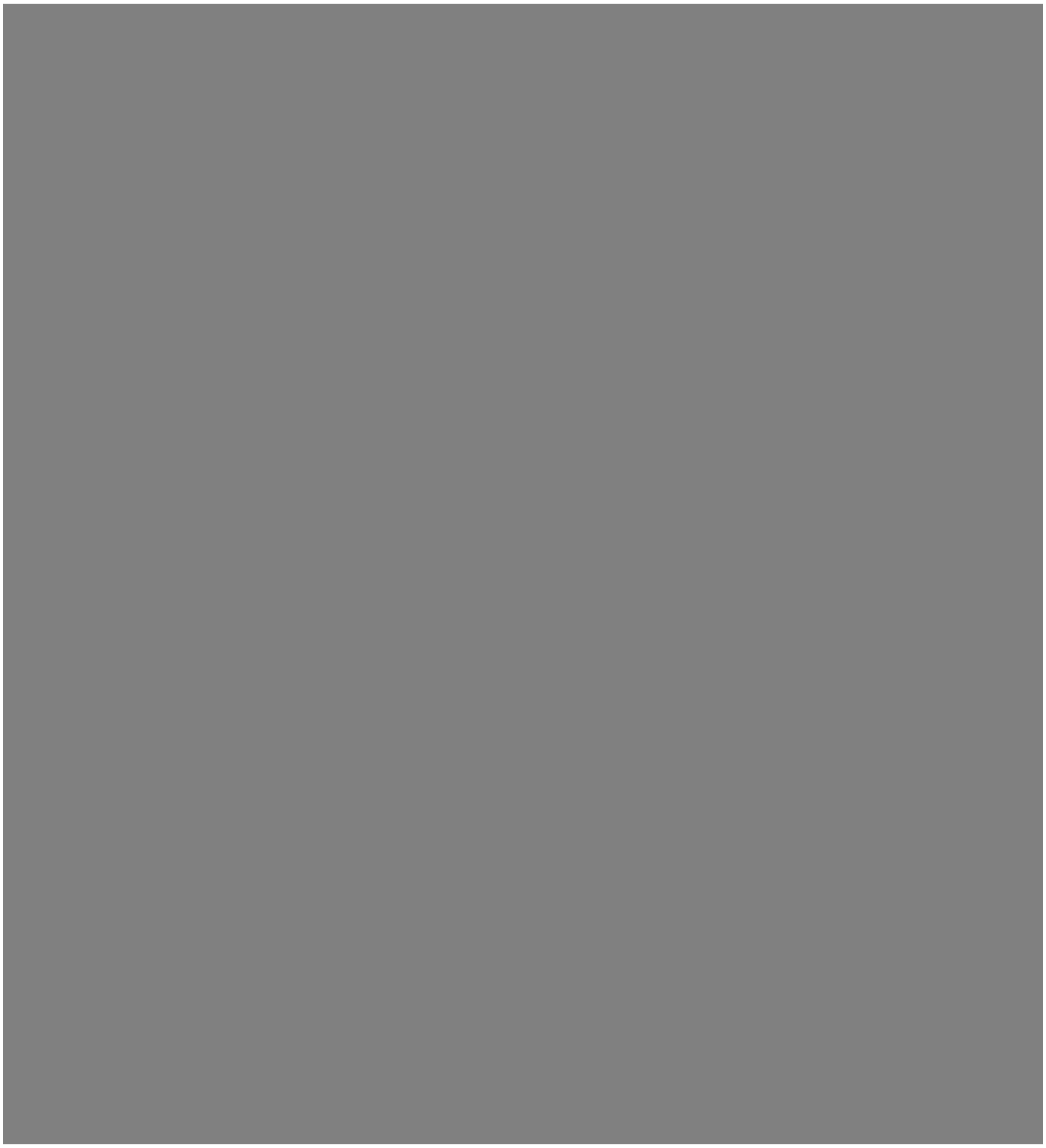
Appendix 6.5

Introduced DNA sequences in the production strain

Diagrams of the xylanase264 expression cassette, which is integrated into [redacted] the *B. licheniformis* chromosome and tables outlining the introduced genetic elements, as well as the full sequence of the DNA that was introduced as a result of the genetic modifications is given below.

[redacted]





Appendix 6.6

Genetic stability of the production strain (Southern blot). Novozymes Report No.: 2012-12765

Genetic stability of the GMM Baking Xylanase used for Baking from production strain HyGe329 assessed by Southern blot analysis.

The purpose of this work is to assess the genetic stability of the GMM Baking Xylanase strain HyGe329. The approach has been to compare DNA prepared from a vial of the master cell bank (MCB) of the *Bacillus licheniformis* strain HyGe329 to DNA from cells isolated after a pilot plant fermentation (referred to as EOP cells) by Southern blot analysis. DNA was prepared from this strain material and the integration pattern of 2 copies of the expression construct for production of the Baking Xylanase in [redacted] *Bacillus licheniformis* was investigated by Southern blot analysis using the internal xylanase HyGe 329 xyl264 coding region as a probe.

Strain material and Southern blot.

The Baking Xylanase master cell bank is termed HyGe329. One vial of HyGe329 was obtained from the Laboratory for Production strains.
Cells from three pilot plant fermentation JBF25, JBF27, and JBF28 were obtained from fermentation pilot plant. The pilot batches were the batches used for manufacturing of the tox material and were performed [redacted] Cells from the pilot plant and production fermentations are collectively referred to as EOP (End Of Production) cells with the batch numbers as identifiers.
Genomic DNA was prepared in the following way:
Cells of HyGe329 and EOP cells from each of the batches were inoculated into each 10 ml Ty broth with Trypticase (Tryptone Yeast Extract Broth) in M-tubes that were incubated at 34-38°C for 1 day.
The formed bacteria cells were harvested and DNA was prepared using a standard DNA purification method (Method references PSL-MSM-0300.01 version 7.0, also filed as Luna: 2003-15731-07).
The DNA was digested with restriction enzyme and a Southern blot was made by hybridization with a DIG labelled probe and chemi-flourorescent detection using CPD star and autoradiography (using X-ray film Amersham Biosciences
Hyperfilm ECL). (Method reference: PSL-SM-3090.01-D version 4.0).

Outline of Southern blot.

The genomic DNA of HyGe329 and EOP cells was digested with the restriction enzyme *EcoRI*. The DNA was applied to a 0.75% agarose gel for the blot.

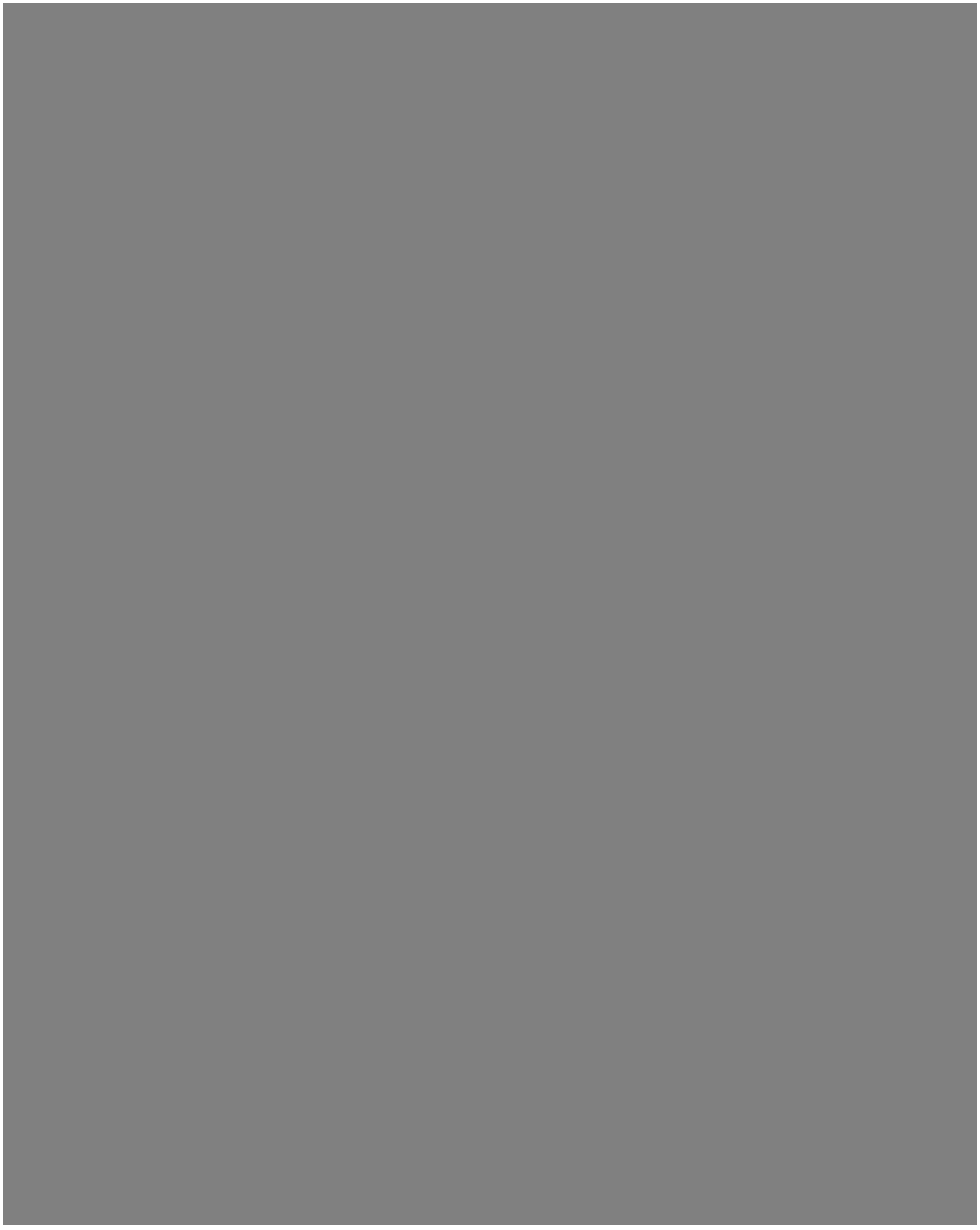
Preparation of DIG-labelled internal Xylanase HyGe 329 xyl264 probe:
The probe used for the Southern blot analysis consisted of a part of the Xylanase HyGe 329 xyl264 coding region. The probe was amplified by PCR from plasmid pBW119 (see Figure 1) according to the procedure described in PSL-SM-3090.01-D using the following primers: [redacted] The size and approximate concentration of the probe was verified by agarose gel electrophoresis (data not shown).

[redacted]

Result and discussion

On the Southern blot (Figure 2) bands hybridizing to the xyl264 probe can be observed in all lanes containing genomic DNA.

The Southern blot bands from JBF25, JBF27, JBF28 and the MCB HyGe329 are observed to be in agreement with the band size expected from the restriction maps [redacted] containing the Xylanase gene (Table: 1 and Fig. 3a and 3b.)



Conclusion

The expected pattern [redacted] from JBF25, JBF27, and JBF28, are identical to the bands pattern of the master cell bank *Bacillus lichniformis* HyGe329, when digestion genomic DNA using the restriction enzyme *EcoRI* , in a Southern blot using the Xylanase xyl264 coding region as probe. This verifies the correct integration [redacted]

It can be concluded that the GMM Baking Xylanase strain HyGe329 is genetically stable.

References for Appendix 6

[redacted]

[4] Yoon, K.H. Yun, H.N. Jung, K.H.; "Molecular cloning of a *Bacillus sp. KK-1* xylanase gene and characterization of the gene product."; Biochem Mol Biol Int 45:337-347 (1998)

[5] Kim, D.-J., Shin, H.-J., Min, B.-H., and Yoon, K.-H. (1995) Kor. J. Appl. Microbiol. Biotechnol. 23, 304-310

[redacted]

[8] Horinouchi, S. and Weisblum, B. (1982). Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide and streptogramin type-b antibiotics. J. Bacteriol., 150, 804-814

[redacted]

[11] Konstantinidis K.T., and Tiedje J.M. (2007) Current Opinion in Microbiology: 10: 504-509

[12] Stackebrandt E. and Goebel B.M. (1994) . International Journal of systematic Bacteriology: p. 846-849