APPENDIX 6

Non-CCI version
Does not include confidential commercial information

Serine protease from *Fusarium oxysporum* produced by a genetically modified strain of *Fusarium venenatum*

Novozymes A/S
August 29th 2014
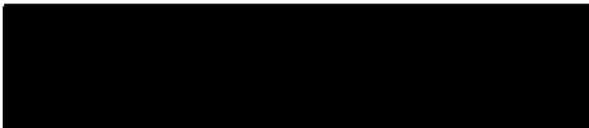
Regulatory Affairs

Appendix 6

Non-CCI version

Elements in Appendix 6 that are to be treated as confidential commercial information (CCI) are marked with a red box in the CCI version. The confidential information has been removed from the non-CCI version. Grey colour has been used for the applied redactions.

Documentation regarding the production strain

1. Detailed description of the construction of the genetically modified production strain

2. Description of general methods

3. Analysis of selected *F. venenatum* strains for production of mycotoxins

4. Annotated DNA sequence

5. Genetic stability of the production strain (Southern blot)

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 *Fusarium venenatum* WTY842-1-11, was derived from the parental strain A3/5 (=ATCC20334), a natural isolate. The taxonomic classification is as followed:

>   Name: *Fusarium venenatum*
>   Class: Sordariomycetes
>   Order: Hypocreales
>   Family: Nectriaceae
>   Genus: *Fusarium*
>   Species: *venenatum*

The identification of the parental strain A3/5 has been confirmed by the American Type Culture Collection (ATCC, Virginia, USA).

Genetic modification

Strains of *F. venenatum* are known to be potentially producers of mycotoxins within the group of trichothecenes, like diacetoxyscirpenol (DAS) (Miller and McKenzie, 2000; Thrane and Hansen, 1995). In fact, under specific inducing conditions, the parental strain may produce DAS and related trichothecenes. Therefore, the gene encoding trichodiene synthase (*tri5*), which catalyzes the first step in the trichodiene biosynthetic pathway, was deleted by means of site-directed gene disruption, thereby rendering it incapable of producing secondary metabolites within the trichothecene biosynthetic pathway.

Construction of the *tri5* deleted host strain

The *F. venenatum* host strain WTY842-1-11 was constructed from strain A3/5

Fig. 1. Strain construction lineage from *F. venenatum* parental strain A3/5 to host strain WTY842-1-11.

Table 1 Overview of relevant genetic elements in plasmid pLC31b. [redacted] [2]Yoder and Christianson (1998). [redacted]

Southern blot analysis confirmed the disruption of the *tri5* gene and insertion of the *amdS* gene in strain WTY-842-1-11.

<u>Verification that the host strain does not produce DAS (diacetoxyscirpenol) and other minor mycotoxins</u>

The host strain WTY842-1-11 was shown unable to produce DAS and related compounds under different inducing conditions and media (Appendix 6.3).

Other minor mycotoxins potentially produced by relevant members of the *Fusarium* genus include i) fusarin C and ii) "butenolide" (4-acetamido-4-hydroxy-2-butenoic acid γ-lactone), a metabolite that has been implicated in animal mycotoxicoses (Desjardins and Proctor, 2007). The host strain was shown not to produce either of these metabolites under different inducing conditions studies, while only traces of fusarin C were detected for the parental strain A3/5 and were only produced under one specific growth condition (Appendix 6.3).

Thus, the host strain does not produce any secondary metabolite of concern.

Absence of production of the secondary metabolites under enzyme production conditions was further confirmed for the serine protease production strain. The result for mycotoxin analysis of production batch PPF 26813 is given in Section A.5 of the main dossier.

6.1.2 Origin and donor of vector and inserts

<u>The enzyme gene</u>
The donor of the ▒▒▒▒▒▒ gene is *F. oxysporum* DSM2672.

<u>The promoter</u>
The promoter is derived from the *F. venenatum* glucoamylase gene (*glaA*) from parental strain A3/5.

<u>The terminator</u>
The transcriptional terminator is derived from the ▒▒▒▒▒ gene of *F. oxysporum* DSM2672.

<u>Vector/inserts</u>
The transforming plasmid pJRoy75 (Fig. 3, section 6.1.3)▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒

The *bar* gene, used as selective marker in plasmid pJRoy75, encodes a phosphinothricin acetyltransferase and is derived from *Streptomyces hygroscopicus* strain ATCC21705.

6.1.3 Introduced genetic sequences

Construction of the recombinant production organism
Cloning of ▆▆▆▆▆▆▆ and construction of pJRoy75 is described below.

The recombinant DNA molecule, pJRoy75 (Fig. 3), was introduced into the host *F. venenatum* strain WTY842-1-11, by incubating protoplasts with ▆▆▆▆▆▆▆▆▆ linearized fragment of plasmid pJRoy75 (does not contain the Amp gene, see Fig. 3). As WTY842-1-11 lacks the *bar* gene, it cannot grow in the presence of phosphinothricin.

Transformants are obtained upon the integration of multiple copies of the plasmid into the chromosomal DNA. Selection of transformants was therefore achieved by growing on a medium with phosphinothricin and subsequent screening for expression of the protease. One transformant that showed high trypsin-like serine protease activity, strain WTY939-8-3, was selected as the final GM production strain.

The general methods used for engineering the strain are described in Appendix 6.2.

It was verified by Southern blot analysis that the production strain contains the ▮▮▮▮▮ gene. As the DNA fragment is integrated by ectopic integration in multiple copies into the genome of the host strain WTY842-1-11 it is not possible to determine the position of integration in the genome or to obtain an accurate genetic map of each individual copy.

As mentioned, plasmid pJRoy75 contains the ampicillin resistance gene (Amp). The ampicillin resistance gene was cut out from the vector and separated from the purified fragment used to generate the transformant production strain. The absence of the ampicillin resistance gene in the final production strain was confirmed by Southern blot.

The fully annotated sequence of pJRoy75 is shown in Appendix 6.4.

6.1.3 Description of the production organism

Identity and taxonomy of production organism

The production organism is a selected strain of *Fusarium venenatum* expressing the *Fusarium oxysporum* serine protease.

The serine protease production strain is further characterized by following phenotypic traits: *tri5-, amds+, bar+.*

Antibiotic resistance gene

There is no ampicillin resistance gene, intact or partial, in the production strain. The bacterial ampicillin resistance gene was cut out from the vector and separated from the purified fragment used to generate the transformant production strain. The absence of antibiotic resistance genes was verified by Southern blot analysis using the relevant antibiotic resistance gene probes.

Stability of the introduced genetic sequences

The presence of the introduced DNA sequences was determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism (Appendix 6.5).

The transforming DNA is stably integrated into the *Fusarium venenatum* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable.

Appendix 6.2

General description of methods
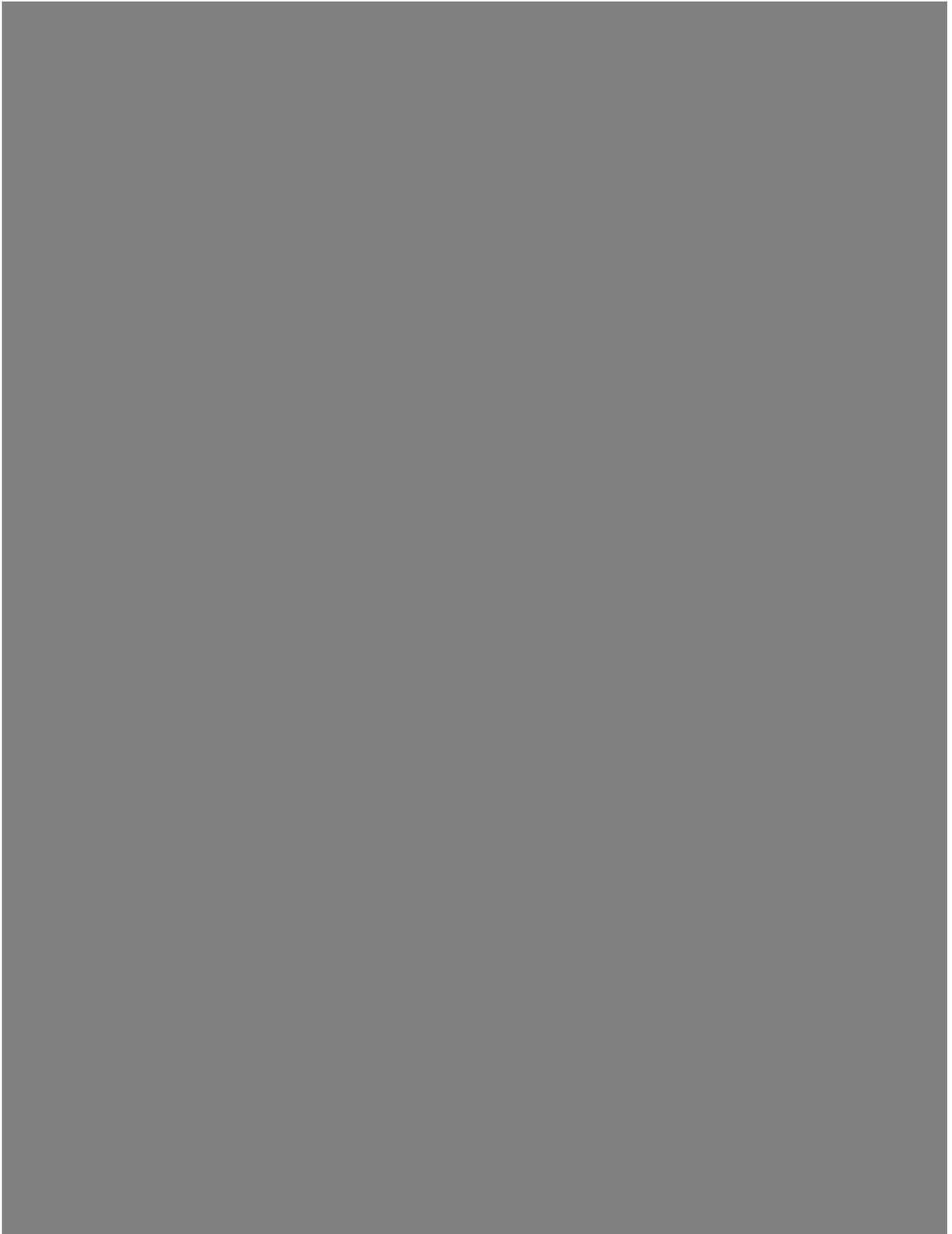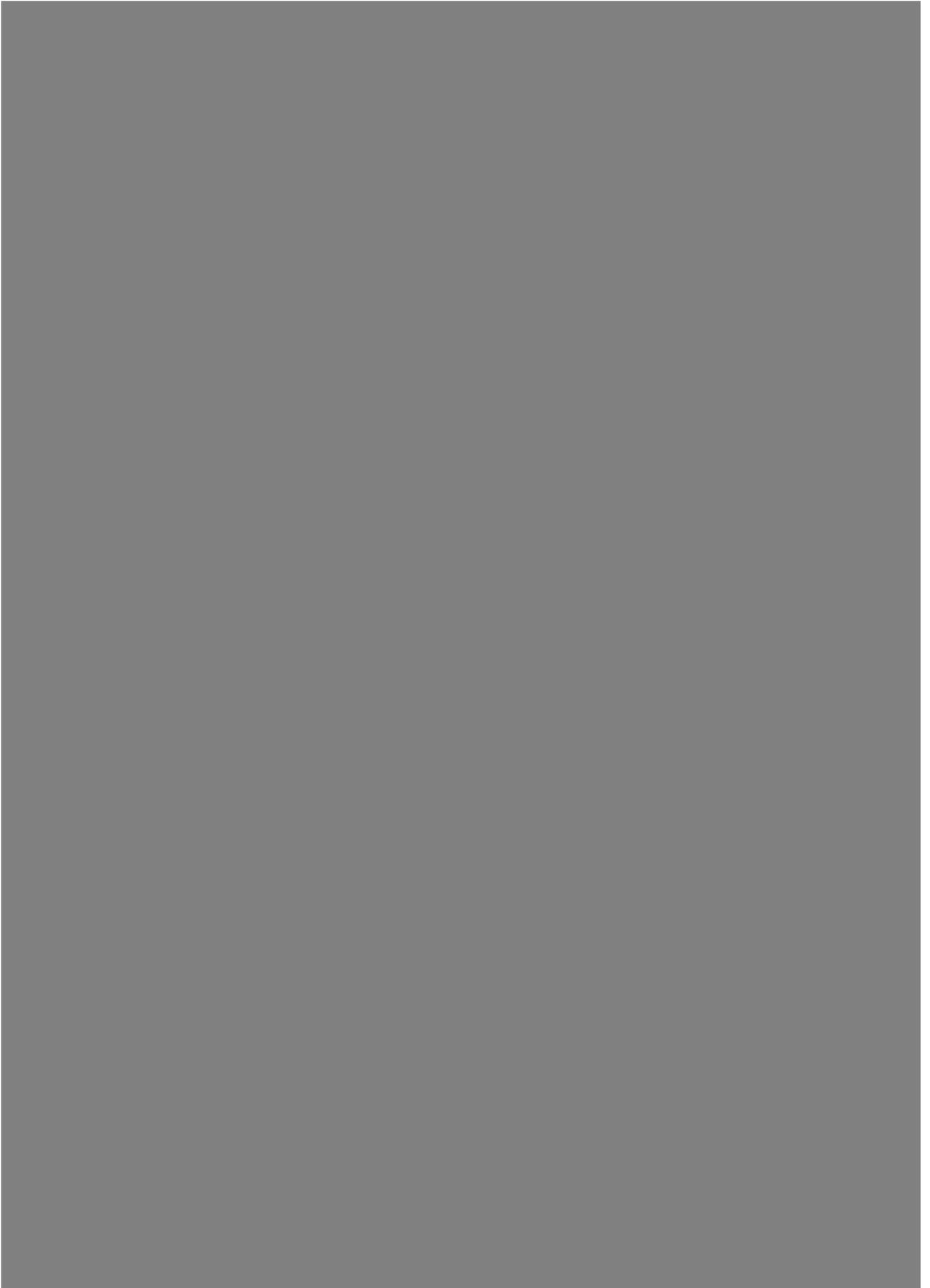
Appendix 6.3

Analysis of selected *F. venenatum* strains for production of mycotoxins

INTRODUCTION

Fusarium venenatum belongs to a taxonomic group of fungi with the potential to produce several toxic secondary metabolites (Thrane and Hansen 1995) such as type A trichothecenes (diacetoxyscirpenol, DAS), and other minor mycotoxins (fusarin C, FUC) and metabolites that have been implicated in animal mycotoxicoses like "butenolide" (4-acetamido-4-hydroxy-2-butenoic acid γ-lactone, Fig. 1; Desjardins and Proctor 2007, BUT).

The present investigation was undertaken to determine the metabolic potential of *F. venenatum* with particular attention to DAS, FUC and BUT. The analysis of secondary metabolites was performed for the *F. venenatum* parental strain A3/5 and the host strain WTY842-1-11 cultured under strong inducing conditions for the production of secondary metabolites.

TAXONOMY AND THE SECONDARY METABOLITE POTENTIAL OF *F. venenatum*

Fusarium species are common saprophytes on plant debris and in soil. Many species are important plant pathogens. The taxonomy of the genus *Fusarium* has been the subject of controversy for many years. Correct identification of strains based on morphological traits has been cumbersome and it is essential when evaluating the potential production of secondary metabolites. In fact, the *F. venenatum* type strain (ATCC 20334) was originally classified as a *F. graminearum* strain, a grass pathogen but both toxin and molecular data confirmed the wrong taxonomic classification (O'Donnell et al., 1998). In a specific European research project, the type species of *F. sambucinum* was studied in detail (Nirenberg 1995). As a result of these investigations, that included a combination of morphological, molecular and metabolic characteristics, the different strains of this species were re-classified in three different new species: *F. sambucinum*, *F. torulosum* and *F. venenatum* (Nirenberg 1995). This classification has been confirmed using DNA fingerprinting methods and has shown that the Novozymes parental strain A3/5 (ATCC 20334) is indeed a *F. venenatum* (O'Donnell et al., 1998; Yoder and Christianson 1998).

Early studies on the potential for secondary metabolite production by *F. sambucinum* and its interpretation in light of the new taxonomical structure of this species concluded that members of the new *F. venenatum* species may include producers of DAS and other DAS-derived trichothecenes, FUC and BUT (Thrane and Hansen 1995).

As an example, *F. venenatum* produces a DAS-derivative compound not identified in *F. sambucinum* while the latter species produces two unique metabolites only identified by HPLC analysis that are not produced by any of the *F. venenatum* strains studied (Thrane and Hansen 1995). The structure of relevant secondary metabolites potentially produced by *F. venenatum* is shown below (Fig. 1).

But

Fig.1. Relevant secondary metabolites potentially produced by *F. venenatum*

MATERIALS AND METHODS

Strains and culturing conditions for the measurement of secondary metabolites
The strains tested in this study are shown in Table 1.

Strain #	Strain Name	Description
1	A3/5	Parental
2	WTY842-1-11	Host

Table 1. *F. venenatum* strains tested in this study.

F. venenatum strain A3/5 (parental) and WTY842-1-11 (host) were revived from storage in 10% glycerol at -140°C by plating onto PDA plates. The two strains were subsequently inoculated onto the following media:
- Raulin-Thom (RT) agar, 5 ml in a 50 ml blue-cap bottle; RT liquid (still), 5 ml in a 50 ml blue-cap bottle; and RT liquid (shake), 5 ml in a 25 ml blue-cap bottle.
- MYRO (Miller et al. 1991) liquid (still) 5 ml in a 50 ml blue-cap bottle; and MYRO liquid (shake), 5 ml in a 25 ml blue-cap bottle
- YES agar, 5 ml in a 50 ml blue-cap bottle; YES liquid (still), 5 ml in a 50 ml blue-cap bottle; and YES liquid (shake), 5 ml in a 25 ml blue-cap bottle (Frisvad 1981)
- PDA agar in standard Petri dish (Samson et al., 2000).

The plates and cultures were incubated at 25°C for 8 days.

Whole cultures (PDA only 10 cm^2) and media-blanks were extracted with 2 x 14 ml ethyl acetate (after 30 min. shaking). The combined ethyl acetate phases were evaporated to dryness in a rotational vacuum concentrator (RVC) from Christ (Germany), redissolved in 500 µl MeOH and filtered through a 0.45 µm syringe filter, ready for LC-MS analysis. This was performed by our standard C18 LC-DAD-ESI+-MS method (Nielsen and Smedegaard 2003), except that the gradient was started at 10% CH_3CN, and that two scan functions were used, one with 29 V and one with 9 V in between the skimmers (for detection of the labile trichothecenes).

Analysis of LC-DAD-MS data
The C18 LC-DAD-ESI+-MS data files were inspected for:
1. MS Scan function
2. Diacetoxyscirpenol (DAS), [M+NH4]+ at m/z 384.15-384.25, RT 11.9 min (see Fig. 2); mono-acetoxyscirpenol, [M+NH4]+, at m/z 342.16-342.23, RT 7.1 min; and triacetoxyscirpenol, [M+NH4]+ at m/z 426.15-426.25, RT 15.8 min. 2.
3. From the UV trace 350-370 nm, fusarin C (poor sensitivity in MS) and analogues, as well as MS scan function 2 for m/z 432 (fusarin C and D) and 416 (fusarin A).
4. All major peaks in the UV and MS traces were matched against the internal metabolite database (~ 600 metabolites).

Detection limits (LOD) were roughly estimated by:
LOD=(IC x Q)/(s/n x noise x recovery), where **IC** is the ion count of the peak apex of the detection ion, **Q** is the quantity of the injected reference standard, **s/n** is the signal-to-noise ratio used to accept a detection (in this case, 10), **noise** is the base line level of the detection ion and **recovery** is the % of the metabolite extracted from sample, set at 50%. In case of SPE this was multiplied by the recovery of SPE column.

RESULTS

Trichothecenes
Strain A3/5 produced DAS on all media and culturing conditions investigated, whereas the host strain WTY842-1-11 showed no indication of production of DAS or other trichothecenes on any of the media and conditions studied. This is in accordance with the previous work published on *F. venenatum* (O'Donnell et al., 1998). From the data in Table 1, it is clear that incubation with shaking stimulated

DAS production. Also, the data indicate that MYRO was the best induction medium for DAS production and for production of trichothecenes derived from DAS in *F. venenatum* (Table 1). These derivatives were only detected under conditions that resulted in high levels of DAS production for parental strain A3/5 (Table 1).

For the host strain WTY842-1-11, no DAS or other trichothecene derivatives were detected even in cultures grown in MYRO medium with shaking (Table 1), demonstrating the lack of potential for the production of DAS in a *F. venenatum* strain with a deletion of the *tri5* gene involved in the biosynthesis of trichothecenes like the host strain (see Annex B2).

The ion chromatograms for cultures grown in MYRO medium with shaking for strain A3/5, WTY842-1-11, and the medium alone as a control are shown below (Fig. 2). A major peak corresponding to DAS is only observed for strain A3/5 (Fig. 2A). The chromatograms of strain WTY482-1-11 and of the medium do not show any trace of DAS, only background (noise) signals (Fig. 2B and C, respectively).

Trichothecene profiling of *F. venenatum* strains grown on different media and conditions				
Strain	**Medium**	**Peak area (ion counts)[1]**		
		15-MAS	**DAS**	**TAS**
A3/5	RT liquid	ND	681	ND
	RT shake	ND	737	ND
	RT agar	ND	534	ND
	YES liquid	ND	3017	ND
	YES shake	82	5513	ND
	YES agar	ND	3119	ND
	MYRO liquid	ND	2650	ND
	MYRO shake	457	13104	1732
	PDA agar	70	4581	ND
WTY842-1-11	RT liquid	ND	ND	ND
	RT shake	ND	ND	ND
	RT agar	ND	ND	ND
	YES liquid	ND	ND	ND
	YES shake	ND	ND	ND
	YES agar	ND	ND	ND
	MYRO liquid	ND	ND	ND
	MYRO shake	ND	ND	ND
	PDA agar	ND	ND	ND

Table 1. Analysis of trichothecene production in *F. venenatum* parental strain A3/5 and host strain WTY842-1-11 grown in different induction media (RT, YES, MYRO and PDA) and conditions. 15-MAS = 15-mono-acetoxyscirpenol; DAS = diacetoxyscirpenol; TAS = triacetoxyscirpenol; ND: not detected. [1]Detection limit for DAS, TAS and 15-MAS (LOD) was 5-20 ppb (parts per billion).
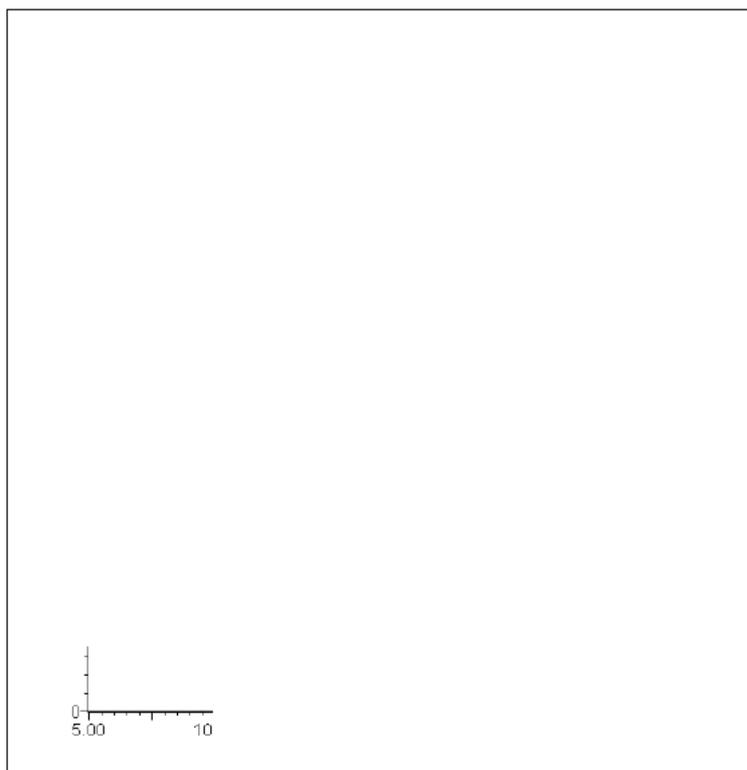
Fig. 2. Extracted ion chromatograms of m/z 384.15-384, [M+NH4]+, comparison of MYRO shake cultures, showing a large (overloaded) DAS peak in strain A3/5 (A), and no detection of DAS in WTY842-1-11 (B) or in the medium (C). The multitude of peaks in B and C do not represent individual metabolites but just noise.

Fusarin C and Butenolide

Fusarin C, as well as fusarin A (tentatively identified) were detected in low quantities in strain A3/5 grown on PDA agar but were not detected in WTY842-1-11 grown on PDA (Table 2). Cultures grown in the other media did not contain detectable quantities of these metabolites for either strain A3/5 or WTY842-1-11 (Table 2). Thus, the host strain does not produce detectable amounts of FUC under strong inducing growth conditions.

Similarly, no butenolide was detected for any of the strains investigated in any of the media/conditions investigated (Table 2). This is consistent with published data showing the lack of production of this secondary metabolite in *F. venenatum* (Thrane and Hansen 1995).

Minor secondary metabolite profiling of *F. venenatum* strains			
Strain	**Medium**	**FUC**	**BUT**
A3/5	RT liquid	ND[1]	ND[2]
	RT shake	ND	ND
	RT agar	ND	ND
	YES liquid	ND	ND
	YES shake	ND	ND
	YES agar	ND	ND
	MYRO liquid	ND	ND
	MYRO shake	ND	ND
	PDA agar	(+)	ND

Minor secondary metabolite profiling of *F. venenatum* strains			
Strain	Medium	FUC	BUT
WTY842-1-11	RT liquid	ND	ND
	RT shake	ND	ND
	RT agar	ND	ND
	YES liquid	ND	ND
	YES shake	ND	ND
	YES agar	ND	ND
	MYRO liquid	ND	ND
	MYRO shake	ND	ND
	PDA agar	ND	ND

Table 2. Analysis of Fusarin C (FUC) and Butenolide (BUT) production in *F. venenatum* parental strain A3/5 and host strain WTY842-1-11 ("WTY") grown in different induction media (RT, YES, MYRO and PDA) and conditions; FUC: Fusarin C; BUT: Butenolide; (+) low level; ND: not detected. [1]Detection limit for FUC (LOD) was 20 ppb (parts per billion); [2]LOD for BUT was 0.5 ppm (parts per million).

CONCLUSION

An investigation of production of relevant secondary metabolites potentially produced by *F. venenatum* was performed. The data generated demonstrate that:

- The *F. venenatum* host strain WTY842-1-11 does not produce DAS and related trichothecenes. The lack of DAS production is the expected result from the inactivation of the biosynthetic pathway that follows the gene disruption of the *tri5* gene performed during strain construction to obtain the host strain WTY842-1-11. The data generated conform to the expected result and demonstrate the lack of potential for production of these compounds in the Novozymes *F. venenatum* strain lineage.
- The *F. venenatum* host strain WTY842-1-11 does not produce any other known mycotoxin or secondary metabolite of concern (FUC, BUT) under the different growth conditions tested
- The parental strain A3/5 produces DAS under all conditions
- The parental strain produced low amounts of FUC under a single growth condition
- The parental strain does not produce BUT

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Appendix 6.4
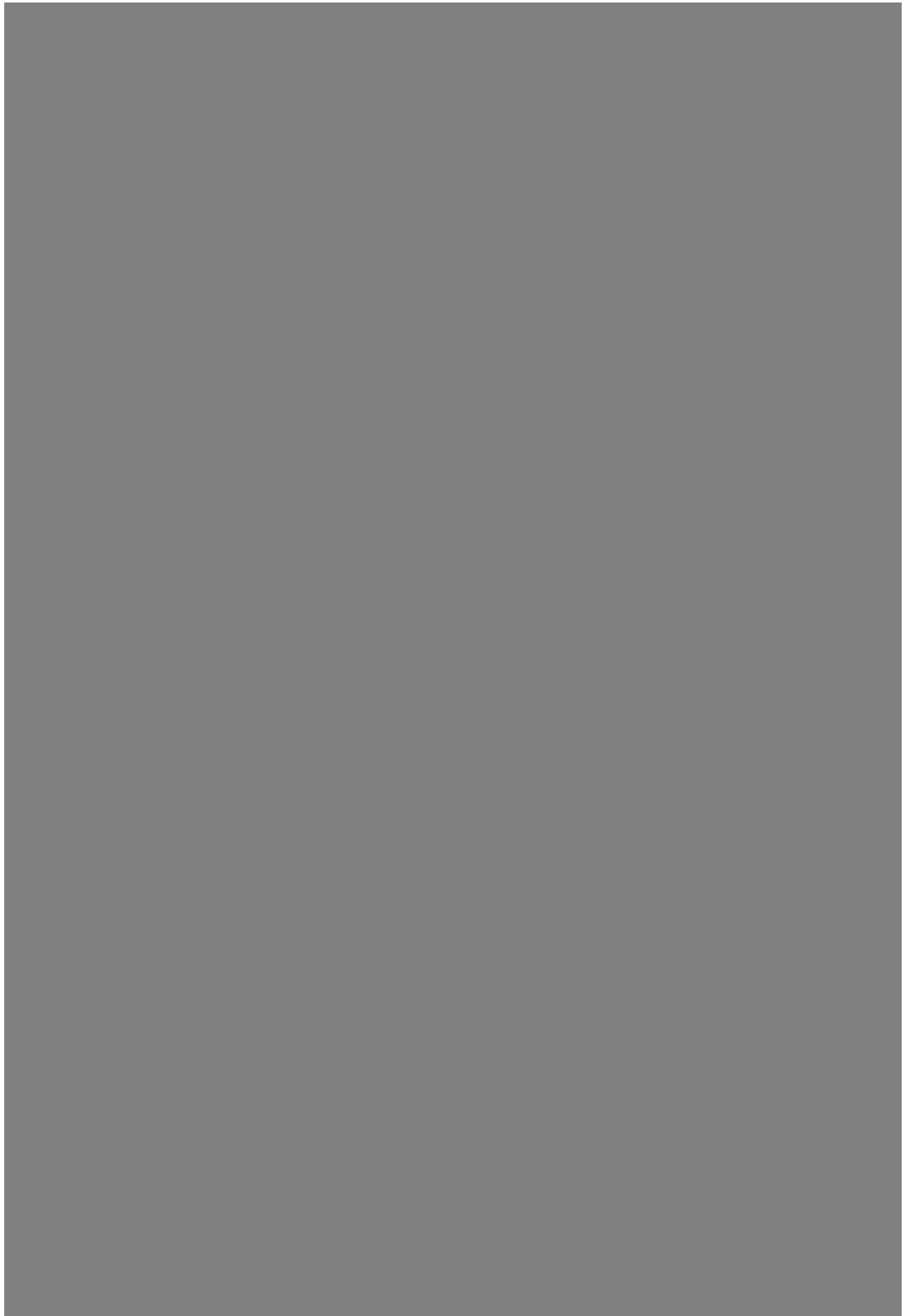
Appendix 6.5

Genetic stability of the production strain, WTY939-8-3

Summary

The genetic stability of the production strain was analysed by Southern blot of genomic DNA obtained from end of production samples, compared to a reference of genomic DNA from the production strain taken from the vial collection.

The Southern analysis of the end of production samples and the reference sample showed no differences in the band pattern, thereby demonstrating the genetic stability of the inserted DNA in the WTY939-8-3 production strain.

Details

The genetic stability of the production strain was analysed by Southern blot of genomic DNA obtained from end of production samples (Table 1). Genomic DNA was isolated from culture suspension (*i.e.*, end of production samples) that were allowed to sporulate and subsequently grow in liquid culture (Fig. 1). This process adds additional generations to the cells used for the analysis allowing the analysis of genetic stability over the intended period of production.

Batch No.
PFF118
PFF119
PFF121

Table 1. Information on the batches used for genetic stability studies.

Identical morphological characteristics were observed for all three end of production samples compared to the production strain reference (colony morphology on plates, sporulation and growth in liquid medium).
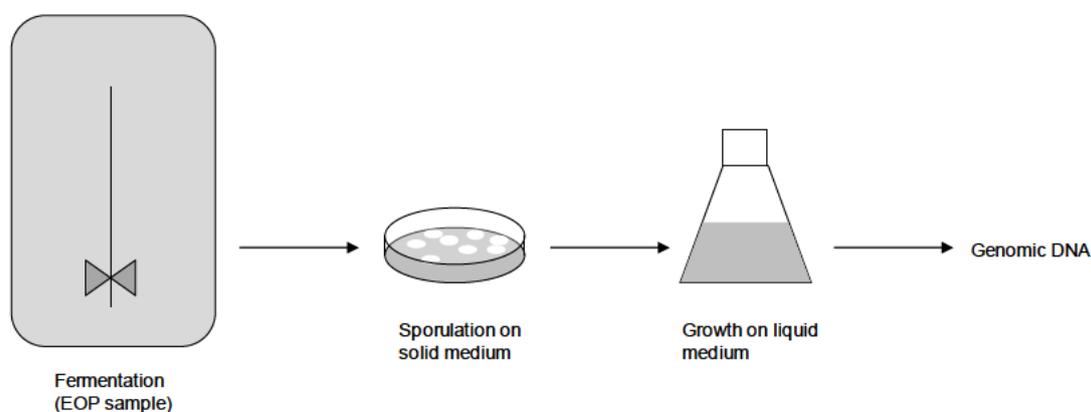

Fig. 1. Overview of genomic DNA sample preparation for genetic stability analysis. A sample from end of production (EOP) from each production batch was taken and used to plate in solid medium to allow for growth of the strain and sporulation after 7 days. Spores were used to produce mycelium by growing in liquid medium 2-3 days. The mycelium was filtered and used for extraction of genomic DNA for Southern blot analysis.

The DNA derived from the end of production samples (Fig. 1) was subsequently analysed by Southern blot analysis, comparing to DNA of the original production strain WTY939-8-3.
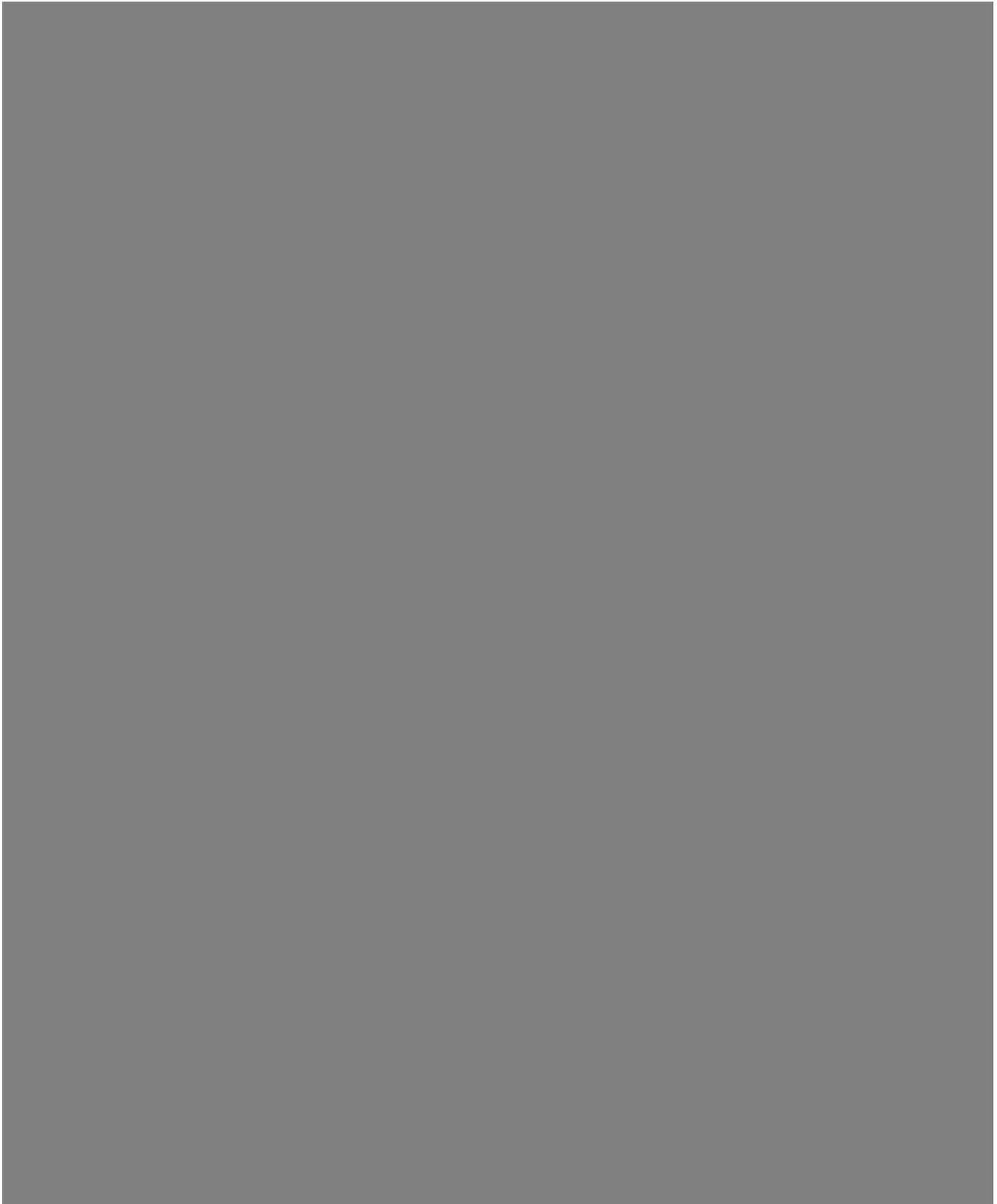
The Southern analysis of the end of production samples and the reference sample showed no major differences in the band pattern (Fig. 2). Thus, the identity in the hybridization pattern of the end of production samples derived from three independent batches and the production strain (WTY939-8-3) demonstrates the genetic stability of the inserted DNA in the WTY939-8-3.

Method for Southern analysis