DRAFT FOR DEVELOPMENT

DD CEN/TS 15790:2008

Animal feeding stuffs — PCR typing of probiotic strains of Saccharomyces cerevisiae (yeast)

ICS 07.100.30; 65.120

BSI British Standards

National foreword

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Comments arising from the use of this Draft for Development are requested so that UK experience can be reported to the international organization responsible for its conversion to an international standard. A review of this publication will be initiated not later than 3 years after its publication by the international organization so that a decision can be taken on its status. Notification of the start of the review period will be made in an announcement in the appropriate issue of Update Standards.

According to the replies received by the end of the review period, the responsible BSI Committee will decide whether to support the conversion into an international Standard, to extend the life of the Technical Specification or to withdraw it. Comments should be sent to the Secretary of the responsible BSI Technical Committee at British Standards House, 389 Chiswick High Road, London W4 4AL.

The UK participation in its preparation was entrusted to Technical Committee AW/10, Animal feeding stuffs.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Date	Comments

TECHNICAL SPECIFICATION

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English Version

Animal feeding stuffs - PCR typing of probiotic strains of Saccharomyces cerevisiae (yeast)

Aliments des animaux - Typage ACP des souches probiotiques de Saccharomyces cerevisiae (levure)

Futtermittel - PCR-Typisierung der probiotischen Stämme von Saccharomyces cerevisiae (Hefe)

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Foreword

This document (CEN/TS 15790:2008) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs – Methods of sampling and analysis”, the secretariat of which is held by NEN.

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Introduction

This methodology is based on specific polymerase chain reaction (PCR) amplification of a genetic sequence for the detection of *Saccharomyces cerevisiae* isolated from animal feed or animal feed probiotic supplement. The aim of this method is to identify authorised probiotic yeast strains. Molecular typing methods and especially PCR amplification based methods used to characterise the yeast strains require high quality high molecular weight genomic DNA. The method of DNA extraction from the yeast must facilitate these requirements.

1 Scope

This Technical Specification defines a polymerase chain reaction (PCR) methodology for the identification of *S. cerevisiae* probiotic yeast strains. Additionally, a method for the extraction of high quality DNA from yeast is suggested.

2 Principle

This method is based upon the amplification of δ elements which are present in the yeast genome. Two primers are used for the PCR reaction, which are a modification of Ness et al. [1]. Distinct patterns are produced for probiotic *S. cerevisiae* strains when separated in agarose gels by electrophoresis. Patterns are visualised under UV light after electrophoresis and ethidium bromide staining of the agarose gel.

The PCR analysis of individual yeast colonies isolated from agar plates involved the following steps:

1. DNA extraction and purification;
2. PCR reaction;
3. Gel electrophoresis;
4. Analysis of results.

Individual and typical colonies can be obtained following growth on appropriate agar media whereby the standard enumeration procedure is recommended that uses yeast extract dextrose chloramphenicol agar (CGYE) [1]. Typical colonies are picked from agar plates to inoculate 10 ml malt extract broth which is cultured overnight at 30 °C in a shaking incubator e.g. an orbital incubator revolving at 100 rpm, or equivalent. The cells are subsequently harvested and DNA is extracted following the instructions from manufacturers when using kits or other appropriate procedures. The DNA extraction procedure is a sequential process of outer cell wall removal, lysis of nuclei, protein precipitation and removal, followed by precipitation of the nucleic acid. An extraction procedure is described e.g. by Hoffman and Winston [2].

3 Reagents

3.1 PCR

3.1.1 Primers

The following primer sequences are used.

Delta 1 modified primer: 5’ CAA ATT CAC CTA TTT CTC A 3’

Delta 2 Primer 5’ GTG GAT TTT TAT TCC AAC A 3’

Stock solutions of each primer are made by diluting in sterile water (3.2.5) to a final concentration of 50 µM and stored at least - 20 °C.

3.1.2 dNTP mix

A 2 mM equimolar stock solution of dATP, dTTP, dGTP, dCTP is made from a dNTP mix set and stored at least - 20 °C.

3.1.3 Buffer

A commercial 10x stock solution of reaction buffer is used. The composition is 100 mM Tris-HCl (pH 9,0), 500 mM KCl, 1 % (v/v) Triton X-100. The buffer is stored at least - 20 °C.

3.1.4 Magnesium Chloride solution

A commercial 10x stock solution is used and stored at least - 20 °C. The composition is 25 mM magnesium chloride ($MgCl_2$).

3.1.5 DNA Taq polymerase

DNA Taq Polymerase with a concentration of 5 U/µl is used and stored at least - 20 °C. Alternative appropriate enzyme preparation may be applicable.

3.2 Gel Electrophoresis

3.2.1 Agarose, molecular-grade agarose free from DNase and RNase contamination.

3.2.2 Molecular weight marker

A 100 bp ladder is recommended.

3.2.3 Tris-Borate-EDTA buffer

Commercially produced 10x TBE diluted with distilled water to 1x (e.g. from Sigma). TBE (1x) is composed of 89 mM Tris Borate-EDTA-buffer, pH 8,3, containing 2 mM EDTA.

3.2.4 Loading dye

A commercially produced 6x loading dye is used. This is composed of 0,4 % orange G, 0,03 % bromophenol blue, 0,03 % xylene cyanol FF, 15 % Ficoll 400, 10 mM tris-HCl (pH 7,5) and 50 mM EDTA (pH 8,0). Appropriate alternative standard loading dyes may be equally applicable.

3.2.5 Water

DNase and RNase free, 18 Ohms, 0,2 µm filtered.

3.2.6 Ethidium bromide

A commercially produced 10 mg/ml solution is used.

4 Apparatus

Usual equipment appropriate for a molecular laboratory and, in particular the following.

4.1 PCR

4.1.1 PCR Tubes

Thin walled DNAse free microtubes appropriate for the thermal cycler that is used.

4.1.2 Pipets and sterile tips

Capable of dispensing between 0,5 µl and 200 µl.

4.1.3 Thermal cycler

An appropriate and reliable thermal cycler which is technically maintained and calibrated.

4.2 Gel Electrophoreses

4.2.1 Horizontal gel electrophoresis system

Including cell and power supply capable of operating at a constant voltage.

4.2.2 Microwave oven

4.2.3 Conical flask

250 ml size for 80 ml to 100 ml of agarose preparation.

4.2.4 Balance

Capable of weighing to the nearest 0,01 g.

4.2.5 Transilluminator

Most appropriate apparatus to visualise the banding patterns in the agarose gel following ethidium bromide staining.

4.2.6 Image analysis system or Polaroid camera

Appropriate system to record and analyse results.

5 Procedure

5.1 PCR reaction

This method has been slightly altered to incorporate modification of the Delta 1 primer [1].

Delta 1 modified primer: 5’ CAA ATT CAC CTA TTT CTC A 3’

Delta 2 Primer 5’ GTG GAT TTT TAT TCC AAC A 3’

For each PCR analysis a premixture containing all the reagents except template DNA is prepared according to Table 1.

Table 1 — PCR reaction mix

	Stock	**Reaction**	**Volume** µl/tube required
Buffer	10x	1x	5
dNTPs	2 mM	200 µM	5
$MgCl_2$	25 mM	1,5 mM	3
Delta 1 modified primer	50 µM	1 µM	1
Delta 2 primer	50 µM	1 µM	1
Taq polymerase	5 U/µl	2,5 U/µl	0,5
H_2O		(to 48 µl)	32,5

The reaction mix is dispensed in 48 µl aliquots into the PCR tubes and kept on ice until the template has been added and the tubes are ready to load onto the thermal cycler.

Add 2 µl of DNA template to final reaction mix (or 2 µl of water (3.2.5) for negative control).

5.2 Thermal Cycle

This step of the procedure is crucial and therefore particular care needs to be taken to ensure that the thermal cycler that is used is reliable with regards to its ability to reproducibly meet the described time temperature cycles. It is recommended to ensure that this is the case by appropriate standardisation procedures applicable for the instrument.

95 °C - 2 min

95 °C - 30 s
45 °C - 30 s
72 °C - 2 min
} 4 cycles

95 °C - 30 s
45 °C - 30 s
72 °C - 2 min
} 30 cycles

72 °C - 10 min

4 °C – 8 min

5.3 Agarose gel electrophoresis

A 2 % (w/v) agarose gel is made with Molecular Biology Grade agarose and 1x TBE. Ethidium bromide is added to a final concentration of 0,1 µg/ml. The running buffer is 1x TBE with a final concentration of 0,5 µg/ml ethidium bromide.

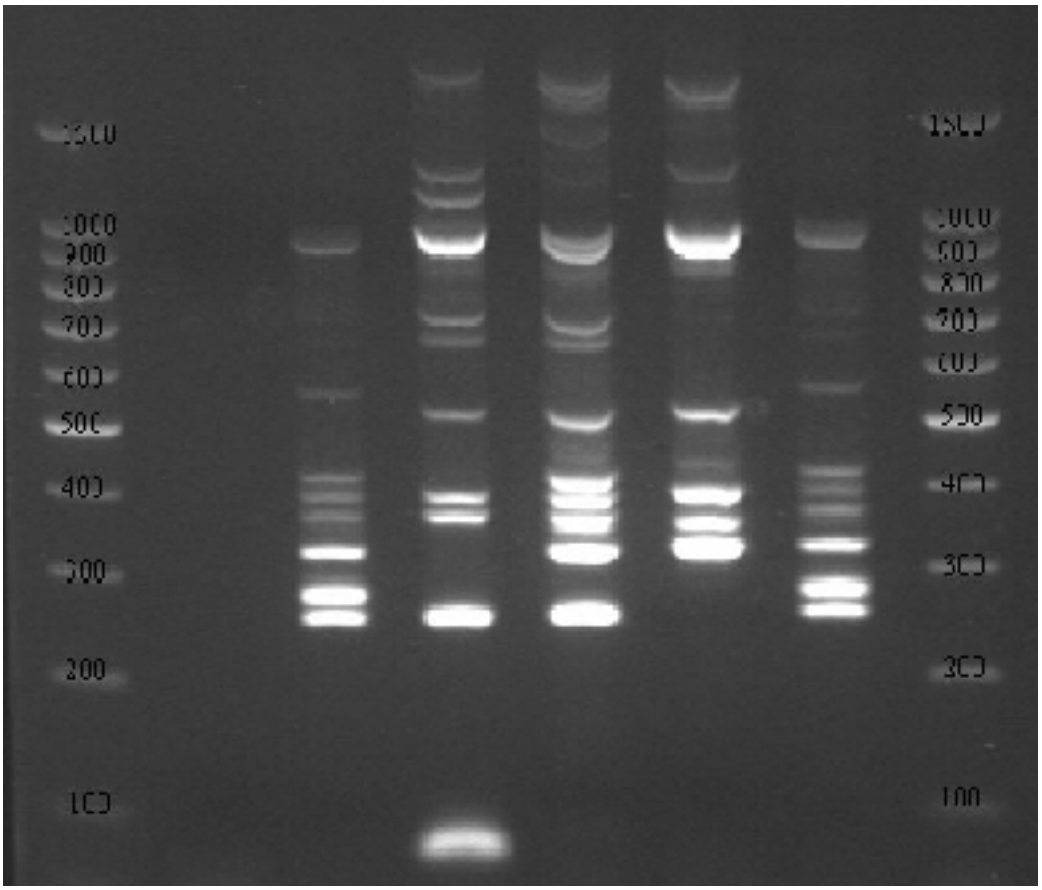
10 µl of loading dye is added to the reaction and mixed. 20 µl of the reaction and dye is loaded into a well for each reaction. The molecular weight marker used is a 100 bp ladder. The product is run at a Voltage of 150 V with a running time between 45 min. and 60 min. The gel is visualised under UV light.

6 Analysis of the results

A reference photo of an agarose gel (Annex A) is provided with distinct patterns from authorised probiotic *S. cerevisiae* strains and a commercially available reference strain (*S. cerevisiae* NCYC 81) for the analysis of the results. The banding patterns as obtained are compared to those in the reference gel on the photo by measuring the size in base pairs (bp) of individual bands using the corresponding molecular weight ladders. It is suggested to record the results by an appropriate camera or image analysis system to facilitate the analysis.

Annex A
(informative)

Example Gel - PCR of probiotic strains of *Saccharomyces cerevisiae*



Key

Lane 1 and lane 8	Promega 100 bp ladder
Lane 2	negative control
Lane 3	APYS[1] CBS 493.94
Lane 4	APYS[1] CNCM 1-1079
Lane 5	APYS[1] CNCM 1-1077
Lane 6	reference strain, NCYC 81
Llane 7	APYS[1] NCYC SC47

Figure A.1 – Example Gel – PCR of probiotic strains of *Saccharomyces cerevisiae*

1 APYS = Authorised probiotic yeast strain.

Annex B
(informative)

Validation data from the European Collaborative trial [4][5]

In the validation study four different samples of animal feeding stuffs containing yeast (*Saccharomyces cerevisiae*) at levels between 10^5 and 10^7 CFU/g by Polymerase Chain Reaction (PCR) were examined. Feeds supplemented with one of four authorised probiotic strains were analysed by seven of nine invited laboratories. The laboratories received 'blind samples' meaning that they did not know before the analysis which yeast strain was in which sample. The laboratories were provided with a photo of reference patterns of DNA fragments characteristic for each strain which formed the reference for the identification of the yeast strains in the samples. All laboratories returned valid results with the exception of one laboratory that had insufficiently separated bands on the agarose gel. The laboratories were asked to perform the analysis on three independently chosen typical colonies as obtained following enumeration of the sample. This was not done by all laboratories as indicated in the table below. The validation was published [4] and [5]. The results are expressed in terms of the number of positively identified colonies compared to the number of target identifications (e.g. 3/3 means that three positive identifications were obtained from three independently analysed colonies which were selected from agar plates).

Blind sample number	**623-632**	**633-642**	**643-652**	**653-662**	
APYS[a]	**CBS 493.94**	**CNCM 1-1079**	**CNCM 1-1077**	**NCYC SC47**	**Reference strain NCYC 81**
7[b]	0 / 1 tested	0 / 1 tested	0 / 1 tested	NT[c]	0 / 1 tested
8	1 / 1 tested	NT	1 / 1 tested	1 / 1 tested	NT
17	3 / 3 tested	1 / 3 tested	3 / 3 tested	2 / 2 tested	1 / 1 tested
19	2 / 2 tested	1 / 2 tested	2 / 2 tested	2 / 2 tested	NT
20	3 / 3 tested	3 / 3 tested	3 / 3 tested	3 / 3 tested	1 / 1 tested
22	NT	1 / 1 tested	1 / 1 tested	1 / 1 tested	NT
26	3 / 3 tested	2 / 3 tested	1 / 3 tested	3 / 3 tested	1 / 1 tested
Correct identification	12 of 12 tested	8 of 12 tested**^**	11 of 13 tested**^**	12 of 12 tested**^**	3 / 3 tested**^**

[a] Authorized probiotic yeast strain.

[b] Bands not sufficiently separated, laboratory was excluded.

[c] Not tested (NT).

^ Reasons for negative identifications: laboratory 17 had some bands in the patterns of 2 lanes missing (CNCM- 1-1079), laboratory 19 had bands missing in the second determination of sample CNCM 1-1079 and laboratory 26 appeared to have mixed-up lanes of samples CNCM- 1-1079 and CNCM 1-1077.

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