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Tracing Bacteria

Marker Aided Detection of Silage Bacteria

Ensiling with selected lactic acid bacteria is widely done. In practice, using commercially or self-produced starter cultures enhances the ensiling process. To develop new ensiling additives and new production methods for starter cultures, diagnostic testing methods are needed to detect the bacteria involved. Classic microbiological methods are mostly unsuitable for this. Molecular genetic techniques are an alternative. As an example, a species-specific assay on determining the presence of frequently used silage bacteria, based on 16S rDNA, was developed.

Preservation of forage material with the aid of lactic acid bacteria (ensiling) is a commonly applied agricultural technique. Ensiling is a naturally occurring process that is often supported by the application of selected bacterial strains which are added directly to the forage in form of liquid cultures or freeze-dried preparations [1, 2, 3]. Various ensiling supplements are available commercially, alongside this, strategies for cheaper on-farm production of starter cultures are underway [4]. Commonly used species in the majority of starter cultures are those such as Pediococcus sp., Enterococcus sp. and Lactobacillus sp., in the latter case often selected strains of *Lact. plantarum, Lact. rhamnosus* and *Lact. buchneri* are applied.

For the development of new solutions for production of such starter cultures, a reliable and reproducible detection of the contributing bacteria species is required. In the cases of *Lact. plantarum, Lact. rhamnosus* and *Lact. buchneri*, classical techniques are often insufficiently specific due to the close relationship of these species and the lack of morphological traits. The species within the group of lactic acid bacteria are often characterised by their type of substrate utilisation, e.g. acid synthesis or the ability to utilise certain carbohydrates. Nevertheless, these methods have several disadvantages e.g. high work load and time requirements.

Since the early 90s, several methods have been developed to identify (micro-) organisms on the basis of their genetic material i.e. their chromosomal DNA. The molecular structure of the chromosomal DNA, the DNA sequence, is specific for every individual and, in contrast to morphological or physiological traits, it exhibits no environmentally-induced variance. Hence, the determination of the DNA sequence enables the absolute classification of individuals to taxonomic groups as well as the detailed analysis of their relationship.

In practice, it is often sufficient to estimate the DNA sequence of a defined region of the chromosome with a suitable intra-specific variability. For this purpose, the bacterial 16S rDNA sequence has been established as a commonly used standard. The 16S rDNA encodes a part of cellular protein-synthesis apparatus (ribosomes). Currently, the 16S rDNA sequence has been estimated for a large number of bacterial species. This data is available for public use online via URL: http:// rdp.cme.msu.edu/html/ [6].

For the detection of single bacteria species, species-specific regions of the 16S rDNA are selected. The DNA between such species specific regions can be amplified in vitro with the aid of an enzymatic reaction, the polymerase chain reaction (PCR, *Fig. 1*), until an amount is reached, which enables an

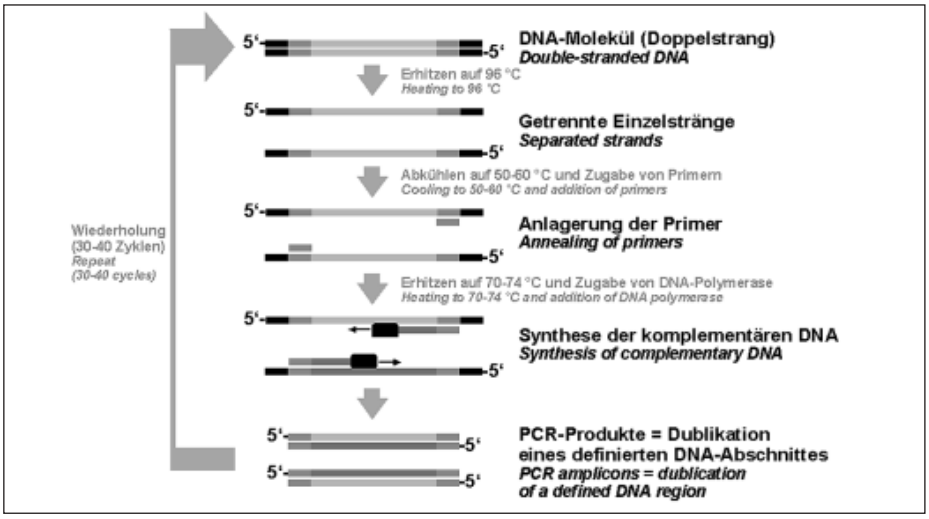

Fig. 1: Scheme of the polymerase chain reaction (PCR) [8]

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Keywords

Silage, lactic acid bacteria, Lactobacillus, PCR assay, 16SrDNA analysis

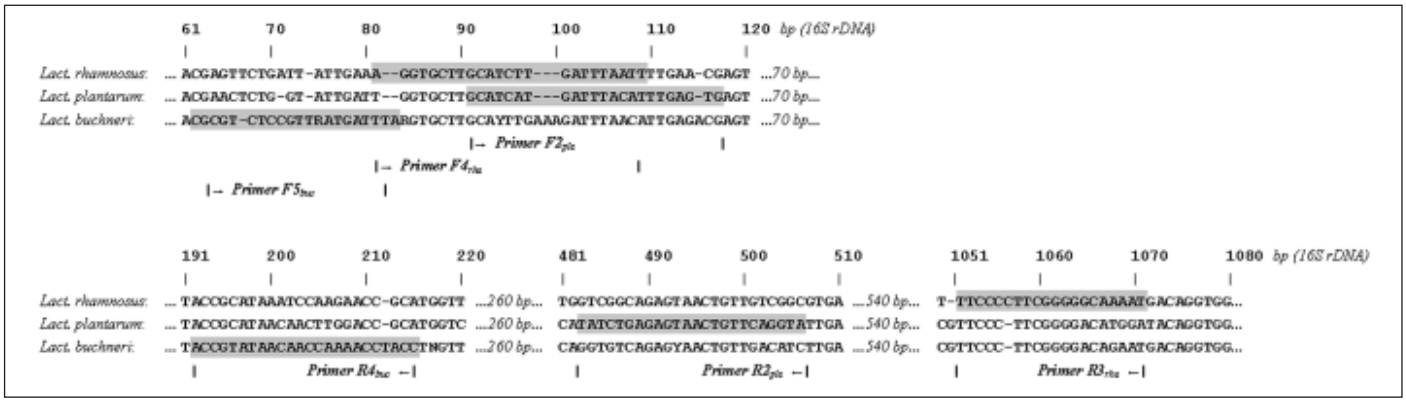

Fig. 2: In silico development of species-specific PCR primers for Lact. plantarum, Lact. rhamnosus and Lact. buchneri based on databank sequences. For this alignment, consensus sequences derived from at least two individual 16S rDNA sequences were used. The primer binding sites are highlighted in grey.

easy detection with optical methods. Furthermore, PCR products with different lengths indicating different species can be obtained through an appropriate selection of starting points of PCR. This strategy facilitates the differentiation of multiple species in one single reaction [7].

Development of a PCR assay for *Lactobacillus plantarum, Lact. rhamnosus* and *Lact. buchneri*

For PCR, short DNA fragments (PCR primers) are required, which are complementary to the desired starting points (*Fig. 1, 2*). To facilitate detection of the species commonly used in starter cultures, the bacteria *Lact. plantarum, Lact. rhamnosus* and *Lact. buchneri* were chosen. From these, variable species regions within their 16S rDNA sequences were selected and used for the design of species specific primer pairs for PCR (*Fig. 2*). A combination of these primers in a multiplex PCR, should amplify the following PCR products depending on the nature of template DNA: a 979 basepair (bp) amplicon indicates the presence of DNA of *Lact. rhamnosus*, a 308 bp PCR product indicates *Lact. plantarum* DNA and a 161 bp amplicon indicates *Lact. buchneri* DNA (Bild 3A).

The expected PCR products were obtained when the multiplex PCR was applied to reference strains of the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (DSZM) (*Fig. 3B*). The PCR amplicons provided a clear differentiation between species, there was no observation of either by-products or false pairings. The same results were also obtained, when the developed primer pairs were applied in a multiplex PCR on mixed DNA templates. Hence, the primers allow strain control even in co-fermentation of multiple bacteria species.

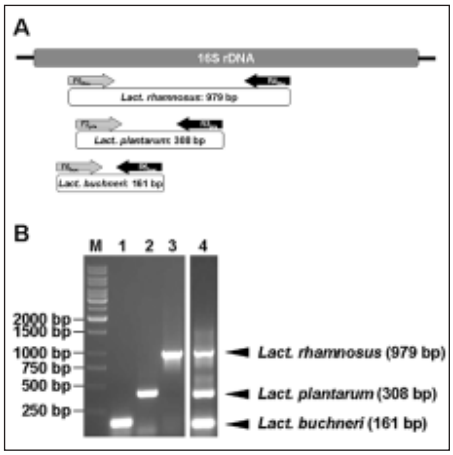

Fig. 3: System for detecting the ensiling bacteria Lact. plantarum, Lact. rhamnosus and Lact. buchneri based on the sequence of the 16S rDNA. (A) Pattern of the theoretical PCR production. (B) Application of the derived PCR primers using DNA preparations of the strains Lact. buchneri DSMZ-20057 (1), Lact. plantarum DSMZ-20205 (2) and Lact. rhamnosus DSMZ-20022 (3) and a mixture of all DNA preparations (4); M marks a length standard.

Conclusion

The identification of microorganisms with the aid of 16S-rDNA-based PCR assays enables a more rapid and reliable detection of single bacteria species than conventional microscopic or physiological techniques. These latter methods often include cultivation steps (e.g. from general to selective media in order to yield a pure culture), which prolong the processing time by several days. In the case of lactic acid bacteria, the classical approaches do not provide a clear differentiation due to the lack of phenotypic variance. In contrast, the PCR-based methods depend not on living cells but on their DNA. Thus, any environmental influence can be excluded. Furthermore, the detection of selected bacteria species can be reached within 3-5 h (depending on the method used for DNA preparation). Hence, the state-of-the-art analyses, which are required for process control of fermentation for the production of starter cultures are enabled. The development of multiplex-PCR assays for parallel detection of multiple bacteria species such as the assay presented in this study will enable the optimisation of fermentation regimes for co-cultivation of those bacteria. Thus, the development of new and also cheaper processes for production of starter cultures will be feasible [9].

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