MOLECULAR METHODS IN FOOD HYGIENE – practical seminar

The practical seminar simulates a model situation you may encounter in real life – we are suspecting that samples of food have been contaminated by potentially dangerous bacteria and we would like to find out which bacteria are present in the samples.

The task is to determine the bacterial species contaminating the food by amplification and direct sequencing of portion of bacterial 16S rRNA gene, using methods of molecular biology. Due to time constraints, some of the steps will be only simulated and explained only theoretically.

The whole workflow would consist of following steps:

(1) isolation of DNA from food sample) – the DNA has been pre-isolated and you will be provided with pure isolate
2) polymerase chain reaction – amplification of a portion of bacterial 16S rRNA gene
3) gel electrophoresis – verification, that the right amplified product is present)
(4) purification of amplified product)
(5) sequencing of amplified product)
6) evaluation and editing of acquired DNA sequences
7) comparison of resulting sequences with GenBank database – taxonomical identification of bacteria from the sample

ad 2) Polymerase chain reaction – PCR

The polymerase chain reaction (PCR) is a simple process, by which we can prepare many copies of a defined target portion of DNA in a relatively short time.

Using PCR, we will amplify (copy many times) ca. 650 bp portion of bacterial 16S rRNA gene. This region of DNA is frequently used for identification of different bacterial species, because its sequence differs significantly among different species (i.e. interspecific variability of the gene). If there is any bacterial DNA in the sample, we should obtain a PCR product, which will be further analyzed in subsequent steps.

To amplify the desired fragment of DNA, we need to mix several components: **PCR water** (pure water, used for filling out the reaction volume), **PCR master mix** (containing DNA polymerase, nucleotides, solution of salts necessary for correct function of polymerase, dye etc.), two **primers** (short oligonucleotides, which flank the target region, and are necessary for polymerase to do the actual „copying“) and the **DNA isolate** from the food sample.

Subsequently, the microtubes containing the mixture are put into a **thermocycler**, a machine, in which the PCR is performed. The reaction itself consists of 25 – 35 cycles, repeating three basic steps: 1) denaturation of double-stranded DNA, 2) annealing of primers, and 3) synthesis/polymeration of a new (copied) strand of DNA. Each of the three steps runs best at a different temperature, so a thermocycler basically is a heated plate (its temperature can be changed very quickly), controlled by a simple computer, which is pre-programmed to change the temperature of the plate in a controlled fashion due to our needs.

Preparation of the PCR mixture:
Take one provided microtube (0,2 ml) per group. Mark the microtube (to identify your sample), and, using provided micro-pipette and pipetting tips, mix following quantities of PCR reagents in it:

6 µl – PCR water
10 µl – PCR master mix
1 µl – primer FF
1 µl – primer FR
2 µl – DNA isolate

After that, close the microtube and put it in the thermocycler.

ad 3) electrophoretic verification of presence of correct amplified PCR product

After the PCR, we need to verify whether we have obtained any product at all, and if so, whether it is of the right size. We will do this using gel electrophoresis, i.e. the DNA fragments will be run through an agarose gel by electric field, and they will be subsequently visualised under UV radiation and their length will be compared to the set of fragments of known length.

Preparation of 1% agarose gel:

1. Put **0,5 g of agarose** into an Erlenmayer flask.
2. Add **50 ml of TBE buffer.**
3. Put the flask in a microwave oven and heat for 2 minutes. When the mixture starts forming bubbles due to boiling, interrupt the heating and mix by careful shaking.
4. After the heating, remove the flask from the oven and cool it carefully under running tap water to ca. 60 °C (a temperature by which you can hold the flask comfortably for several seconds).
5. Add **3 µl Midori Green** stain using a micropipette and mix by stirring.
6. Prepare the electrophoretic apparatus, put the comb in and pour the heated gel in it.
7. Remove bubbles by pipette tip.
8. The gel takes some 20 minutes to solidify.

Horizontal agarose gel electrophoresis and DNA visualisation

1. When the gel is solid, put it in the chamber with TBE buffer (the gel has to be covered) and remove the comb.
2. Using a micropipette, put **3 µl of size standard (ladder)** in one of the pits.
3. Put respective samples (5 µl) in following pits. Manipulate the micropipette carefully, so you won't pierce the gel.
4. Apply the electrical field – constant voltage 130 V for 20 - 30 minutes.
5. Once the electrophoresis is over, transfer the gel to a UV-transilluminator and evaluate the result under UV-light. Because of safety, it is necessary to look at the gel through the plastic cover of the instrument.

ad 4) purification of PCR product

Obtained product of PCR has to be purified, i. e. we have to get rid of unused primers, nucleotides, salts and other reagents, because they would interfere with the sequencing reaction.

ad 6) evaluation and editing of obtained DNA sequences

An external company provides us with raw data from the automatic sequencer, which have to be further processed. We need to evaluate the quality of the obtained data, edit them if necessary, and prepare a final representative sequence of each sample/fragment. There is a number of softwares available for this purpose, we will use MEGA (free to download from http://megasoftware.net).

ad 7) comparison of resulting sequences with database and taxonomic identification of bacteria

Our resulting sequences will be compared to sequences stored in GenBank database. This database contains more than 183 million publicly available DNA sequences from more than 100 000 distinct organisms.

The MEGA software contains an interface which enables us to compare our sequences with the GenBank directly. The output tells us, which sequences stored in GenBank are the most similar to our sequence we want to identify. We look for 100% identity, which means that our sample contained DNA from respective bacterium.