Doctoral theses

INVESTIGATION OF LACTIC ACID BACTERIA WITH DEVELOPED COLORIMETRIC METHOD

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The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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1. INTRODUCTION

It can be said without exaggeration, almost a revolution has gone on in the last 30 years in the microbiology. However it is less know and appreciated its effects open up new perspectives in the medicine, agriculture and also in the food industry. The new, chiefly molecular biology methods and techniques discovered that much of the form of life enhance in the microdimensions. Diversity of viruses, bacteria, fungi, algae and protozoans exceed the diversity of the better known and abounding macroorganisms, flora and fauna.

The new microorganisms, which are waiting for the discovery, are the repository of the genetical versatility and metabolism. The discovery of this will has far-reaching consequences and will impact on several area of practical life, such as the enhancement of the agricultural production, food preservation, improvement of food safety and quality, new, efficient medicines, recycling of the wastes and disposal of the environment damaging pollutions.

It can be said generally the microorganisms in the foods and raw materials, when they grow up, decrease the quality of the product, make spoilage, however in several cases they contribute to the character of the product, moreover they increase the self-life and the enjoyment value of the product and they have beneficial effect on the health of the consumer. The food microbial investigations cover the raw materials, the processing line and the end product, however in point of view of the food chain the investigation of the microbial state of the environment and monitoring of the microbial changes during the transport and distribution is necessary, because of the safety and appropriate food products.

Several microbiological methods, which are applied still in our days, are developed more than 100 years before. Millions of the investigations are made with this traditional culture methods, nevertheless these are time-, work- and material-consuming and the result could be estimated often just after several days (DEÁK, 2006a; DEÁK, 2006b). Because of this the traditional culture methods, where the living cell number are counted, are not appropriate to obtain the microbiological results in a proactive, preventive, for the demand of modern quality control appropriate quick and efficient way. The moving force of the modernization of the methods is the increasing problem of the microbiological safety all over the world and the methods should adapt to the changing conditions of food production, -process and distribution. The detection of the damaged, non-reproducible, but living

microorganism is also important. These changed demands require we get the results faster, from more samples with less work, cheaper and even so more informative. For the satisfaction of these demand the microbiology can call the tools of several associate-science and -specialty, for example the analytical chemistry, physical chemistry, enzymology, immuno-biochemistry and biotechnology, moreover the results of molecular biology and genetic engineering, the sensor- and instrument-development, tools of the electronics and the computing technology to help. The modern microbiology methods can detect the microbe-biomass selectively and determine the growth and the metabolites of the microbes with the help of these methods (FARKAS, 1998).

The sensitivity of instrumental methods, namely the lower limit of the detectable cell concentration still lags behind significantly from the classical culture methods, and the increasing of the sensitivity of these methods entails usually the increasing of the time demand of the investigations. In the further development great emphasis has placed on the increasing of the sensitivity of the rapid methods and the enhancement of the specificity, as well as on the detection of the viable but non culturable cells.

The lactic acid bacteria, including the lactobacilli are close to human for thousands of years, which is not surprising, since these microorganisms can be found in our environment in several niches, for example on the surface of plants, in the soil and in the human intestinal tract, in this way they contribute significantly to the support of the health immune system and help the digestion process. The *lactobacilli* exert their beneficial effect by their primary and secondary metabolites. The mankind used these beneficial properties already before our era to ferment vegetables, meat and milk, in such way prolong the self-life of the foods. These microorganisms increase to a great extent the digestibility and the enjoyment value of the food products, beyond the prolongation of the storage time.

Over these beneficial activities, the daily intake of a food product, which contains *Lactobacillus* in an appropriate amount, so called probiotic foods, improves the functions of our immune system and decrease the risk of the occurrence of several diseases.

„There is not any other group of bacteria, like the lactic acid bacteria, which has so diverse relationship with the humans. Because of it is important and necessary to better know they.” (DEÁK, 2005).

For this reason the aims of my work are to select bacteriocin producer strains from the *Latobacillus* genera with molecular biology method and to develop a colorimetric rapid method based on the measuring of dehydrogenase activity to measure the inhibitory activity of the bacteriocin, which method enables the quick detect of the living cell numbers of microorganisms.

1.1. AIMS

My research aims were:

- To adapt a quick, alternative colorimetric method based on the measurement of dehydrogenase enzyme activity for analyzes viable cell number of the *Lactobacillus* strains, which is also applicable for measuring the inhibitor activity of bacteriocins.
- To determine the parameters of the newly developed method (such as pH, media, incubation time), the achievement characteristics for its possible validation and its comparability with traditional methods measuring also viable cell numbers.
- To devise DNA isolation method for detecting bacteriocin coding genes, and to optimize the PCR assay.
- To isolate the protein-like inhibitor by gelchromatography from the supernatant of strains, which contain the PCR-identified bacteriocin (plantaricin) coding genes and to determine its molecular weight.
- To certify the inhibitor activity of the isolated bacteriocin.

2. MATERIALS AND METHODS

2.1. Microorganisms used for the experiments

Lactobacillus strains:

Lactobacillus acidophilus N2, *Lb. casei* subsp. *casei* DMF 30120 154, *Lb. casei* 2107, *Lb. casei* 2752, *Lb. casei* 2756, *Lb. casei* 2763, *Lb. casei* Shirota, *Lb. curvatus* 2768, *Lb. curvatus* 2770, *Lb. curvatus* 2771, *Lb. curvatus* 2775, *Lb. delbrueckii* subsp. *bulgaricus* B397, *Lb. fermentum* D13, *Lb. fermentum* DT41, *Lb. paracasei* subsp. *casei* DMF 30136 SF1, *Lb. paracasei* subsp. *paracasei* DMF 30134 05, *Lb. paracasei* subsp. *paracasei* 2749, *Lb. paracasei* subsp. *paracasei* 2750, *Lb. plantarum* DMF 30131 01, *Lb. plantarum* 2108, *Lb. plantarum* 2142, *Lb. plantarum* 2741, *Lb. plantarum* DSM 9843 299v, *Lb. plantarum* VE56, *Lb. rhamnosus* DMF 30105 VT1, *Lb. rhamnosus* GG, *Lb. sakei* DSM 20017 *sakei*

Bacterium strains for control:

Thermoplasma acidophilum DSM 1728, Thermobifida cellulosilytica DSM 44535,

Escherichia coli DH10B

2.2. Broths

Syntetic, laboratorial broths: de Man, Ragosa, Sharpe (MRS) tápleves, MRS agar, MRS soft agar, milk broth

Natural media: jerusalem artichoke broth, red beet broth

2.3. Applied methods

The cell number, the growth and the sensitivity against the inhibitor materials of the examined lactobacillus strains were examined by plate-poor method, agar diffusion method, turbidity method (on microtiter plate, at 630 nm), and the further-developed 3-(4,5-dymetyl thiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT) colorimetric method.

For the molecular biological analyses, DNA in adequate quantity and quality from the examined microorganisms was recovered by a combined method of a Wizard and a QuickGene-mini80 DNA isolation. The DNA fragments amplified by the adapted PCR technique were detected by polyacrylamide gelelectrophoresis.

According to the presence of bacteriocin coding genes, proteins in supernatant containing the PCR-selected *Lactobacillus*, were fractionated by gelchromatography (semi-synthetic Molselect G-25, 40-120 μm). The inhibitor activity against *Lb sakei* DSM20017 strain of the separated fractions was controlled by turbidity measurements on microtiter plate. The molecular weight of the inhibitor fraction was determined by SDS-PAGE.

3. RESULTS

My research aim was to develop a quick, alternative method base on the measurement of dehydrogenase enzyme activity for analyses of the *Lactobacillus* stains, which is suitable to measure the number of viable cells and theirs sensitivity against different inhibitors, which needs less time than the traditional method based on cultivation.

For the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide MTT colorimetric assay originally developed for mammalian cells studies, the first step was to determine the optimal concentration of the tetrazolium bromide (8-9 mg/l MTT) in the measuring cell concentration range of the analysed *lactobacillus* species (10^7-10^8 cell/ml) and the incubation time.

When I was working out this method my experience was similar to the literatures as several chemicals may influence the reduction of MTT and the applied MRS and the Jerusalem artichoke broths for lactic acid bacteria growing caused tetrazolium bromide reduction and therefore it disturbed the accuracy of the measurement. So it was concluded that in every cases it was necessary to remove the broth from the surface of the bacteria cells before adding tetrazolium bromide and the following incubation.

During optimization the 2 hours and 37 ^{o}C incubation was ideal, regarding that my aim was to develop a quick method. During the use of this method it has been discovered that the dehydrogenase activity of the lactobacillus cells depend on not only the number of viable cells and the growing speed, but also depend on the strains, and even the composition of the applied cell-grow medium also has an influence on it. So it can be declared that prior special calibration is needed for the developed MTT method to evaluate the accurate viable cell number in the case of all *Lactobacillus* strains and it is important to pay attention to the applied broth as well.

Furthermore the reductive ability of the tetrazolium bromide showed decreasing tendency as the pH decreased. Under controlled circumstances and parameters, there is close correlation between the concentration of the created formazan crystals and the number of viable cells.

This developed colorimetric method is suitable to determine the viable cells of the lactobacillus in less than 4 hours.

I developed a quick method, which can carry out in 2 ml eppendorf tube (9mg/ml MTT, 2 hour-incubation, 37 ^{o}C) and in 96-well microtiter plates (8 mg/ml MTT, 2 hour-

incubation, 37 ^{o}C), so it needs less chemicals and it is also preferable that it can be used for 32 samples in three parallels.

My further aim was to select *lactobacillus* strains from strain-collection based on their bacteriocin production by the means of molecular biology methods. To achieve this aim, I worked out a new isolation method to obtain the DNA of the lactobacillus strains. The combination of the Wizard and the QickGene-mini80 DNA isolation method was the novelty of this developed method, which improved the efficiency in quantity and purity of the DNA isolation from the bacterium cells.

I optimized the PCR method for identifying *lactobacillus* strains and detecting the plantaricin bacterocin coding gene. Firstly I had to determine the optimal parameter of the PCR reaction (optimal concentration of the primer and the template DNA, optimal primer-binding temperature, PCR cycle number), which is necessary for the single template DNA amplification.

100 ng template and 0.8 μM primer concentration resulted in the strain-specific detection of lactic acid bacteria (IFL-IRL primers) gave the sharpest DNA fragment bands on gels of electrophoretic measurements. The next step was to choose the proper temperature of the primer binding or annealing. Applying the previously optimized reaction mix composition, the analyses were achieved on 52-68 °C primer binding temperature. Results showed that the most acceptable primer binding temperature was 59 °C. Under these adjusted parameters, I used 33 cycle-reaction period.

For the plantaricin gene-specific PCR reaction, the 100 ng template and 0.8 μM primer concentration (PlnA1-PlnA2) was the most suitable. The optimal primer binding temperature was 55 °C, and the 33 cycle –reaction period was also usable.

I managed to select successfully several strains **(**01; 2108; 2142; 2750; 2756; 2768; 2775; 299v; *sakei*; VE56; N2), which has plantaricin coded gene. The supernatants of the selected species were fractionated on column by chromatographical method, and from the fractions, which the growth of the test microorganism (*Lactobacillus sakei* DSM 20017) inhibited, the presence of bacteriocin was detected by SDS-PAGE and its molecular weight (1.43 kDa) was also determined. By the means of agar plate technique, I do proved that the detected protein was the responsive for the growth inhibitor of the *Lactobacillus sakei* DSM 20017 strain.

4. NEW SCIENTIFIC RESULTS

1. **I successfully adapted the MTT method primarily developed for human cells by Mosman (Wang et al., 2007) for counting viable cell number of Lactobacillus strains. I defined the optimal parameters:** bacterium cell concentration, MTT concentration, incubation time, pH effect, which all influences the correlation between the cell number and the formazan concentration. The method was miniaturized on microtiter plate (Hegyi et al., 2012).
2. I established that the quantity of the evolving formazan (dehydrogenase activity) were significantly different between certain strains, which was significantly influenced by the composition of the broth for individual strains as well. **I certified that differences in dehydrogenase activity also existed in case of different carbohydrate based media, which provided the same growing speed of bacteria.**
3. **I certified that the developed method was not sensitive for dead cells. In view of calibration curve of the analysed Lactobacillus strain cell concentration grown on MTT and the optical density of the given sample, the ratio of the live and dead cell can be determined.**
4. **The developed protocol is suitable for analyses of the sensitivity of certain strains to those components, which inhibit their growth**. Furthermore, this method is adaptable to determine the minimal concentration of the inhibitor, which was confirmed by use of nizin.
5. I adapted PCR to detect *Lactobacilli* on the base of their DNA. **I proved the presence of plantaricin-coding gene by PCR, the bacteriocin production of those strains, which possessing the gene. I also confirmed the expression of the gene and determined the molecular weight of the gene-product as well.**

5. CONCLUSIONS AND SUGGESTIONS

According to my results, I observed that the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphfenyl-tetrazolium-bromide (MTT) colorimetric method can be adaptable to determine quickly the live cell number of *Lactobacilli,* the effect of inhibitor components, and the effect of different environmental parameters on the cell viability.

The method can be also used for quick analyses of several foods, mostly functional products, which have to contain exact number of live cells till the consumption in order to be able to provide the expected beneficial impact (probiotics). Furthermore, its usage could be more important for a quick detection of the spoilage and pathogen microorganisms, which is possible with some modification of parameters of the method.

Due to the changing dietary habits of people, claims are increased to food with natural, favourable nutritional value and long preservative time, treated by a moderate preservative procedure and made with natural preservatives.

Lactic acid fermentation is an excellent method for the production of these kinds of food products, at which besides there is acidic effect antimicrobial materials (bacteriocins) can be produced contributing to their long self storage and supporting favourable nutritional characteristics.

So the selection of the lactic acid bacteria according to their bacteriocin production has a great importance. To achieve this selection, a well-developed molecular biological method is essential, by the help of which the presence/lack of the bacteriocin coding gene can be detectable. It is also important to make sure in the expression of the bacteriocin coding gene under the certain technological circumstances. As further research aims full examination of those environmental factors which induce and enhance the bacteriocin production of strains should be significant by the help of the developed method.

6. PUBLICATIONS RELATED TO THE SUBJECT OF THE PHD THESIS

Publication in journal

Articles with impact factor:

ZALÁN, ZS., HUDÁČEK, J., TÓTH-MARKUS, M., HUSOVÁ, E., SOLICHOVÁ, K., **HEGYI, F.**, PLOCKOVÁ, M., CHUMCHALOVÁ, J., HALÁSZ, A. (2011): Sensorically and antimicrobially active metabolite production of *Lactobacillus* strains on Jerusalem artichoke juice. *Journal of the Science of Food and Agriculture,* 91 (4), 672–679. IF_{2011}: 1,436

HEGYI, F., ZALÁN, ZS., HALÁSZ, A. (2012):
Improved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay for measuring the viability of lactic acid bacteria. *Acta Alimentaria*, 41 (4), 506-512. IF_{2012}: 0,475

Articles without impact factor:

LÁSZTITY, R., HALÁSZ, A., ZALÁN, ZS., NAGY, A., **HEGYI, F**., GELENCSÉR, É. (2010): Probiotikus tejsavbaktériumok felhasználhatók-e a kenyérgyártásban? Sütőiparosok, Pékek. 57 (2), 25-26.

Publications in conference proceeding

Abstracts in Hungarian:

HEGYI, F., ZALÁN, ZS., HALÁSZ, A., (2010): 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólium-bromid (MTT) alkalmazása tejsavbaktériumok enzimaktivitásának mérésére. XXXIII. Kémiai Előadói Napok Szeged, 2010. október 25-27, program és előadás-összefoglalók, p. 195.

Proceedings in foreign language:

HALÁSZ, A., ZALÁN, ZS., NAGY, A., **HEGYI, F.**, GELENCSÉR, E. (2009): Can probiotic LAB strains be used to improve nutritional quality of bread? *5th International Congress FLOUR-BREAD '09 Proceedings*, p. 30-38. ISBN 978-953-7005-21-4

ZALÁN, ZS., **HEGYI, F.**, HALÁSZ, A. (2012): Improvement of whole grain breads microbial safety. *6th International Congress FLOUR-BREAD '11 Proceedings*, p. 22-29. ISSN 1848-2562

Abstracts in foreign language:

HALÁSZ, A., ZALÁN, ZS**.,** NAGY, A., **HEGYI, F**., GELENCSÉR, E. (2009): Can probiotic LAB strains be used to improve nutritional quality of bread. *5th International Congress FLOUR-BREAD '09 Abstract book* (ISBN 978-953-7005-19-1), p. 30.

HEGYI, F., ZALÁN, ZS**.**, HALÁSZ A. (2009): Development of Colorimetic Assay for Measuring of Bacteriocin Activity. Poszter- in: EFFoST 2009 Conference: New Challanges in Food Preservation Abstract CD, [P275].

HALÁSZ, A., BARÁTH, Á., MÁTRAI, B., ZALÁN, ZS., **HEGYI**, **F.**, NÉMETH M. E., KING-DOBOZI, SZ. (2010): From SCP to probiotics - Challenges for foodchemists. *Women Chemists and Innovation, October 20-22, 2010- Keszthely, Hungary, Programme and Book of Abstracts*, p. 25. ISBN 978-963-9970-08-3.