CORVINUS
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Faculty of Food Science

PhD thesis

SELECTION OF LACTIC ACID BACTERIA FOR INHIBITION OF GROWTH OF SPOILAGE YEASTS

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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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The Doctor Council of Lifescience Area of Corvinus University of Budapest have designated in the decision on 10. July 2008 the untermentioned appraising Board for the conduction of open debate

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1. INTRODUCTION

The conserving of the acquired, produced nutriment in consumable condition for long time is the ancient desire of the mankind. The main reason of the deterioration of the food products beside the forthcoming physical and chemical changes in the food is the outgrowing of the undesired microorganisms and the negative changes what they cause. Suitable storage conditions can eliminate this problem partly, but in case of long time storage is needful to preserve the product in an other way. It can be solved with physical (e.g. heat treatment, reduction of water activity, irradiation), physical-chemical (e.g. salting, curing, smoking) and chemical (e.g. chemical additives) methods, from which many change the physical consistency and the chemical composition of the product and by this its naturalness. However there are increasing consumer demand on chemical preservatives free, minimally processed, so called "natural", fresh food products. A natural solution for it is the biopreservation. In biopreservation, storage life is extended and/or safety of food products is enhanced by using natural or controlled microflora and/or their antibacterial products. The most investigated and applied microorganisms for biopreservation are the lactic acid bacteria, which are a large group of beneficial, gram-positive, non-sporing, acid tolerant rod or cocci shape, aero tolerant anaerob bacteria that have similar properties and all produce lactic acid as an end product of the fermentation process, and they are widespread in nature and are also found in our digestive systems. Lactic acid bacteria have been used in food production and preservation for centuries, unknowingly, and only in the last 150 years are they used intentionally to develop the taste and consistency as well as to ensure the safety of foods. The great number of published articles in the theme of lactic acid bacteria in these days shows that they hide several newness yet, in particular if we consider that the species show varied, different property in the same genus, and within the species the strains too. The one of the most important genus of lactic acid bacteria is the genus of *Lactobacillus*. The members of this genus, beside that they are a natural, important and beneficial constituent of certain food, and because of it they are frequent members of the protective and starter cultures, have got proven probiotic properties. Because of it the investigations with this genus have got advantaged importance.

The main spoilage organisms of our foods are the moulds and yeasts. The effect of lactic acid bacteria on fungi was already investigated by several researchers, nevertheless this effect is

influenced by the diversity of the species, strains, they different behaviour and the mere environment, where this effect is investigated.

1.1. Aims

The objective of my PhD thesis was to determine the growth, the fermentative property and the antimicrobial metabolite production (acid, hydrogen peroxide) of 10, earlier pre-selected *Lactobacillus* strains in a synthetic and a natural media.

To study the inhibitory effect of the investigated, good antimicrobial metabolite producer strains against yeasts.

To reveal the inhibitory mechanism of the *Lactobacillus*, which have shown good antifungal activity, in special consideration of the proteinaceous inhibitory metabolites, and in case of detection I set myself as an aim to purify and determine it.

I take into my aims to form mixed cultures from the efficient antifungal strains and investigate their inhibitory activity.

I set myself as an object to investigate the effect of individual *Lactobacillus* strains, the mixed cultures and the purified inhibitory component against yeasts in vegetable juice model, and in this way to found an appropriate starter culture or natural antifungal component for practical use.

2. MATERIALS AND METHODS

2.1. In the investigations applied microorganisms

Lactobacillus strains:

Lactobacillus casei subsp. casei 154, *Lb. casei* Shirota, *Lb. curvatus* 2768, *Lb. curvatus* 2775, *Lb. paracasei* subsp. *casei* SF1, *Lb. paracasei* subsp. *paracasei* 05, *Lb. paracasei* subsp. *paracasei* 2750, *Lb. plantarum* 01, *Lb. plantarum* 2142, *Lb. rhamnosus* VT1 (strains were obtained from Dairy Institute of the Agricultural Faculty of Perugia and from Dairy and Fat Technology Department of Institute of Chemical-Technology of Prague)

Yeast strains:

Candida famata DMF 1001, *Candida glabrata* CBS 138, *Kluyveromyces marxianus* var. *lactis* DMF 1004, *Saccharomyces cerevisiae* 2880

2.2. In the investigations applied media

Synthetic, laboratory: de Man, Rogosa, Sharpe (MRS) broth

Natural: Jerusalem artichoke broth

2.3. Applied methods

I have investigated the growth of the microorganisms with **cell colony counting**, in case of the lactobacilli with agar plate pouring (MRS agar), in case of the yeast with spreading (PDA agar), and with **measuring of turbidity**, on microtiter plate, at 630 nm.

I have measured the organic acids with **isotachophoresis** from the cell free supernatant of the given media, after one night incubation.

I have investigated into the phosphate buffer excreted hydrogen peroxide with **enzymatic coloured reaction**, after pre-incubation in the given media.

I have investigated the direct effect of the secreted antimicrobial substances with **colony-test method**, the effect of the excreted metabolites was measured both at acid and at neutral pH (pH

6,5), and the unique effect of the organic acids and the hydrogen peroxide, respectively, by **agar well diffusion-test** and **microtiter plate-method**. I applied these methods also for the investigations of the yeast-inhibitory properties of lactobacilli.
I have done the extraction, the purification of the inhibitory component with an **adsoption-desorption method**, where the producer cells were used as adsorbent. I have proven the protein nature of this component with **thin layer chromatographic method** and protein hydrolyser **enzymatic treatment**. The determination of the molecular weight of the purified substance was done by **gel-electrophoresis**.

I have measured the amount of the produced histamine by lactobacilli with **ELISA kit** test.

The forming of the mixed cultures was done according to the results of the **cross-streak method** and the **agar well diffusion method**, considering the effect of the strains on each other.

I have used Jerusalem artichoke vegetable juice as food-matrix for the investigation of the inhibitory effect of *Lactobacillus* strains, the formed mix cultures, their supernatants and the purified proteinaceous component from the point of view of the food industrial applicability. Into this food-model the test yeast strains was added beside the *Lactobacillus* strains, the supernatants and the proteinaceous component, respectively, and the yeast growing was observed at 25 °C incubation.

3. RESULTS

One of aim of my PhD thesis was to investigate the antimicrobial metabolite production, the organic acid and hydrogen peroxide excretion of some *Lactobacillus* strains in laboratory and natural media. The acid-profile of the investigated strains in the different media could be determined according to the measured acid concentrations. Generally lactic acid was produced by the strains in the greatest amount in both media, however certain strains showed a lower lactic/acetic acid ratio, moreover in the Jerusalem artichoke media was produced, according to its amount, more acetic acid in some cases. By the ratio of lactic acid:acetic acid the fermentative character of the strains can be determined, and according to this 3 strains, the *Lb. casei* subsp. *casei* 154, the *Lb. plantarum* 2142 and the *Lb. paracasei* subsp. *paracasei* 2750 showed heterofermentative property both in the synthetic MRS and the natural Jerusalem artichoke media, while the other 7 investigated strains showed homofermentative property. I have measured the hydrogen peroxide excretion of the strains in phosphate buffer after a pre-propagation in the different media, and I found that after the pre-propagation in the synthetic, laboratory media they produced 2-7 times more hydrogen peroxide quantitatively than in case of the Jerusalem artichoke. On the other hand I did not find relationship between the fermentative character and the hydrogen peroxide production of the strains. I have established about the metabolites production, that the amount of metabolites considerably depend on the given media. I investigated the individual effect of the produced organic acids, namely the lactic and acetic acid, and the individual effect of hydrogen peroxide against the yeasts, with media, which were complemented with these metabolites, according to the concentration, what the investigated strains produced, and I have found their considerably dependence on pH.

I investigated the yeast-inhibitory effect of *Lactobacillus* strains both directly (colony-test method) and according to their excreted substances (agar well diffusion-test) and I established, that in case of the direct effect the strain *Kluyveromyces marxianus* var. *lactis* was well inhibited, while in case of the *Candida* strains this inhibition varied, however on the *Saccharomyces cerevisiae* they had not shown effect at all directly.

On the other hand I did not find relationship between the fermentative character and the degree of inhibition. I obtained in the case of the neutral supernatant greatly varied inhibitory effect, which depend on the producer strains. Although by the investigation of direct effect I found more

powerful inhibition, the supernatant on neutral pH also showed inhibition against the growth of yeasts, which indicate other substance than the organic acids and the hydrogen peroxide, and this was confirmed also by the considerably inhibitory effect of the concentrated supernatant.

I have purified successfully a substance in the case of the strain *Lactobacillus plantarum* 2142 with the adsorption-desorption method, which substance showed growth inhibitory effect against certain test yeast, but expressly against the strain *Candida glabrata*, and I have proven the protein nature of this substance with thin layer chromatography and enzymatic treatment. I have determinated the molecular weight of this protein and I found that this antifungal component is a peptide below 11 kDa.

The biogenic amine production of *Lactobacillus* strains is important from the point of view of food safety, with special regard to histamine. I have measured the histamine production of the investigated strains both in the laboratory and the natural media and I found that 4 strains have produced histamine and only one of them, the strain *Lb. paracasei* subsp. *paracasei* 2750 showed histamine production in both media and only in relatively great concentration, since it was lower than the threshold limit.

I had got as an object to form mixed cultures, which have got greater inhibitory effect against yeasts than the strains individually. For this purpose I investigated the effect of the strains against each other both directly and with their supernatants, from point of view of the possibly produced bacteriocins. From these results I formed 7 mix cultures, which were investigated for their inhibitory effect against yeasts and were compared to the individual effect of strains. I have found, that the mix culture of strains *Lb. curvatus* 2768 and *Lb. casei* subsp. *casei* 154 has got synergistic inhibitory effect against the test yeasts.

I investigated the effect of the individual strains, the mixed cultures and the supernatants (both at acidic and neutral pH) of them against yeasts on Jerusalem artichoke juice, which was used in this experiments as a food-model, from the point of view of the food industrial applicability of their antifungal property.

I have established, that beside the direct application of the *Lactobacillus* strains the supernatants from the Jerusalem artichoke broth have shown anti-yeast effect at neutral pH too, and the purified antifungal protein also showed inhibitory effect in the vegetable juice.

3.1. New scientific results

1. I have determined the organic acid production of 10 authentic lactic acid bacteria, the strain *Lactobacillus casei* subsp. *casei* 154, *Lb. casei* Shirota, *Lb. curvatus* 2768, *Lb. curvatus* 2775, *Lb. paracasei* subsp. *casei* SF1, *Lb. paracasei* subsp. *paracasei* 05, *Lb. paracasei* subsp. *paracasei* 2750, *Lb. plantarum* 01, *Lb. plantarum* 2142, *Lb. rhamnosus* VT1, and according to the ratio of the produced lactic and acetic acid I have established their homo- or heterofermentative character: from the investigated strains 7 (the strain *Lb. casei* Shirota, *Lb. curvatus* 2768, *Lb. curvatus* 2775, *Lb. paracasei* subsp. *casei* SF1, *Lb. paracasei* subsp. *paracasei* 05, *Lb. plantarum* 01, *Lb. rhamnosus* VT1) proved to be obligate homofermentative and the other 3 (the *Lactobacillus casei* subsp. *casei* 154*, Lb. paracasei* subsp. *paracasei* 2750, *Lb. plantarum* 2142) heterofermentative. I have found, that the intensity of the hydrogen peroxide production is not in connection with the fermentative character.
2. I have selected successfully lactic acid bacteria strains with inhibitory activity against yeasts (*Lactobacillus casei* subsp. *casei* 154, *Lb. curvatus* 2768, *Lb. plantarum* 01, *Lb. plantarum* 2142) and I have formed mix culture (*Lactobacillus casei* subsp. *casei* 154 and *Lactobacillus curvatus* 2768), which shows synergistic antifungal activity.
3. I have proven the production of an antifungal proteinaceous substance, below 11 kDa molecular weight in the case of the strain *Lactobacillus plantarum* 2142, which substance was not described so far.
4. I have found from the experiments, where the strains, the mix cultures and their supernatants were investigated against yeast on vegetable juice as food-matrix, that beside the direct application of the *Lactobacillus* strains the supernatant from the fermented Jerusalem artichoke juice have got anti-yeast effect too.

4. CONCLUSIONS AND SUGGESTIONS

- I have studied during my investigations the acid, hydrogen peroxide, other inhibitory components and histamine production of 10 *Lactobacillus* strains in different media and I have found, that the amount of produced metabolites considerably depend on both the producer strain and the given media. Drive from this the activity of the antifungal components depend also on the strain and the environment, and in this way the *Lactobacillus* strain, which we want to apply for food preservation, have to be investigated in that media/food-matrix, where we want to apply them in the future.
- After the investigation of the acid production of *Lactobacillus* strains I have determined the acid profile of this strains and from the ratio of the lactic and acetic acid their fermentative character. The ratio of the produced lactic and acetic acid on different media can be a good marker of the fermentative character of lactic acid bacteria, namely the homo- or heterofermentative property of this bacteria.
- Certain microorganisms, so thus also the *Lactobacillus* strains, can both inhibit and help the growth of each other. From my results it can be seen, that with formation of mixed cultures one can develop a starter culture with extended efficiency. On the other hand it is suggested to investigate further this mixed cultures from the point of view of the production of metabolic components, which one was produced more intensively and what influenced this.
- I have detected, purified and partially characterized a proteinaceous substance in case of the strain *Lb. plantarum* 2142, which has shown significant inhibitory effect against the test-yeasts also by itself. It can established from my results and from that not numerous publication, which relate also proteinaceous substance with anti-yeast effect from lactobacilli, that also these microorganisms can produce specific substance, which inhibit the growth of yeasts. These components, after further investigations, can give a good alternative on the field of the yeast inhibition, within the scope of the biopreservation. Therefore the particular indentification of these substance should be done and investigations in different food-matrices, and suggested the determination of the genes, which play role in the production of these components and the enhancement of the productivity of the producer strain.

PUBLICATIONS CONNECTED WITH THE SUBJECT OF THE DISSERTATION

Publication in review

Article in review with IF:

BARÁTH, Á., HALÁSZ, A., NÉMETH, E., **ZALÁN, ZS.** (2004): Selection of LAB strains for fermented red beet juice production. *European Food Research and Technology,* 218 (2) 184-187. p.

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ZALÁN, ZS., BARÁTH, Á., HALÁSZ, A. (2003): Romlást okozó élesztőgombák gátlása tejsavbaktériumok törzsekkel. *Lippay János-Ormos Imre-Vas Károly Tudományos Ülésszak Összefoglalók,* 174. p.

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