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POTENTIAL OF *LACTOBACILLUS PLANTARUM* CCM 3627 AND *LACTOBACILLUS BREVIS* CCM 1815 FOR FERMENTATION OF CEREAL SUBSTRATES

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ABSTRACT

Lactobacillus is the most representative strain in a group of lactic acid bacteria, which perform an essential role in the preservation and production of wholesome foods. Lactic acid fermentation is the oldest traditional method for preparation of fermented vegetables, meat products, dairy products and cereal foods. Cereal grains are considered to be one of the most important sources of dietary proteins, carbohydrates, vitamins, minerals and fibre for people. The main exploitation of cereals is to prepare sourdough, which is a mixture of wheat, rye or other cereal flour with water and contains yeasts and lactobacilli. The basic biochemical changes that occur in sourdough bread fermentation are acidification of the dough with organic acids produced by the lactobacilli and leavening with carbon dioxide produced by the yeast and the lactobacilli. Acidification perhaps initiate enzymatic processes of proteins and phytates degradation. Lactobacilli produce various enzymes which make flavour precursors, improve of mineral bioavailability or degrade celiac active peptides, because some species of lactobacilli produce specific peptidases during growth, which are capable to hydrolyze hardly cleavable, celiac-active proline-rich peptides. Microbial fermentation with selected strains of lactobacilli may be new alternative approach for modification of gluten by hydrolysis. In this paper are described growth characteristics and intracellular aminopeptidases activities of *Lactobacillus plantarum* CCM 3627 and *Lactobacillus brevis* CCM 1815. Work was focused on characterization of the lactobacilli for potential usage as a starter culture in further fermentation experiments.

Keywords: sourdough fermentation; *Lactobacillus*; growth; acidification; proteolysis

INTRODUCTION

Cereals are source of saccharides, dietary fibre, proteins, mineral elements and vitamins required for human health. Cereal goods also contain antinutrients such as phytic acid and are poor in essential amino acid content **(Kocková and Valík, 2011)**. Microbial fermentation has been reported as a tool which can improve mineral availability of cereal goods by reduction of phytate, enhancing the flavour by microbial production of organic acids and releasing of essential amino acids by proteolysis **(Kohajdová and Karovičová, 2007)**. In addition, nutritional features of cereals can be modified by proteolysis by sourdough microflora, which may lead to decreasing of amounts of celiac-active peptides (**Nionelli and Rizzello, 2016**).

Celiac disease is a chronic inflammatory disorder triggered by ingestion of gluten proteins and similar proteins from rye and barley, in genetically susceptible individuals (**Gobbetti et al., 2014**). Gluten proteins are consist of prolamins – gliadins and glutenins. Prolamins are proline-rich molecules with compact structure, difficult to hydrolyze. Prolamins of wheat are also known as gliadins, secalins are prolamins of rye and hordeins are barley prolamin fraction. From technological point of view, gluten proteins are irreplaceable, because they are responsible for viscosity and elasticity of dough and gas retention during fermentation process (**Osella et al., 2014**).

Rollán et al. (2010) describe sourdough fermentation as one of the oldest traditional biotechnological process of bread-making. Sourdough is the mixture of flour and water, spontaneous fermented by presented metabolically active microflora. Microorganisms naturally associated with sourdough are yeasts and lactic acid bacteria at a ratio of 1:100. **Gereková et al. (2011)** characterize lactic acid bacteria as group of functionally and genetically releated bacteria, belonging to the genera *Leuconostoc*, *Lactococcus*, *Pediococcus*, *Lactobacillus*, *Weissella Tetragenococcus*, etc. *Lactobacillus* genus is frequently observed and prevalent strain in sourdough. Lactobacilli are Gram-positive, non-sporing, rod-shaped and non-motile bacteria. According the hexose metabolism, they may be divided into two groups. First group is

homofermentative lactobacilli utilizing glucose via the Embden-Meyerhof pathway. They produce more than 85% of lactic acid as the main product. Heterofermentative lactobacilli metabolise hexoses via the phosphoketolase pathway and produce only 50% of lactic acid. They produce also acetic acid, formic acid, ethanol, carbon dioxide and aldehydes. Strains of lactobacilli which ferment glucose via heterofermentative pathway are adapted to live in microaerophilic conditions **(De Vuyst and Neysens, 2005; De Vuyst and Vancanneyt, 2007)**.

Microbial acidification is crucial for activation of enzymes, which may improve nutritional properties of fermented cereal food. Phytases and endogenous cereal proteases are activated under acid conditions. Wheat and rye flour contain ca. 1% of phytate, which bind cations of calcium, iron or magnesium. These complexes are not accessible for hydrolysis above pH 5.0 and bioavailability of presented essential microelements is reduced. Cereal phytases are active in the pH value range from 3.5 to 5.0. **(Gänzle, 2014)**. In the same way, acidic conditions play key role for initiation of proteolysis during fermentation process. Lactic acid bacteria shift pH value under 4.0 and activated endogenous cereal proteinases, which promote degradation of protein molecules into the various sized peptides. Peptides are transported across the bacterial cell membrane into the cytoplasm. There are presented various intracellular peptidases, which complete the proteolysis and liberate free amino acids **(Gobbetti et al., 2014)**. Most lactic acid bacteria associated with sourdough do not produce extracellular peptidases **(Moroni et al., 2009)**. Lactobacilli produce also proline specific peptidases and their activities vary at strain levels. Increased levels of amino acids was observed after cereal fermentation. The composition of lactic acid microflora of dough strongly affect on the concentration of amino acids in dough after fermentation process. Selected strains of bacteria using as a starter culture for cereal fermentation may be new biotechnological tool for producing gluten-low wheat and rye products **(Gänzle et al., 2008)**.

In the last decades, potential of sourdough lactic acid bacteria as „cell factory" of proteolytic enzymes was investigated. **Di Cagno et al. (2002)** and **Di Cagno et al. (2004)** reported producing a sourdough bread that is tolerated by celiac sprue patients. Their studies showed the ability of selected sourdough lactobacilli to hydrolyze of wheat prolamins effectively and extensively. **Rizzello et al. (2007)** described successful study of sourdough bread preparation using selected lactobacilli and fungal proteases.

The Codex Alimentarius claims that "gluten-free" label may also be used for products containing no more than 20 ppm of gluten proteins. Products with no more than 100 ppm of gluten can be labeled as "gluten-low" **(Walter et al., 2014)**.

The aim of this study was to evaluate growth of *L. plantarum* CCM 3627 and *L. brevis* CCM 1815, characterized by increase of optical density of cells in cultivation medium in time, increase of dry cell weight and change in pH value of cultivation medium. Aminopeptidases activites were determined from crude enzyme extracts per volume of solution in μkat.dm^{-3}.

MATERIAL AND METHODOLOGY

Bacterial strains and growth conditions

Growth characteristics of *Lactobacillus plantarum* CCM 3627 and *Lactobacillus brevis* CCM 1815 were investigated. Pure lyophylized cultures of both lactobacilli strains were obtained from Czech Collection of Microorganisms (Brno, Czech Republic). Lactobacilli were grown in de Man, Rogosa, Shrape medium (MRS medium) M369 (HiMedia, India) containing pepton 10 g.dm^{-3}, beef extract 10 g.dm^{-3}, yeast extract 5 g.dm^{-3}, dextrose 20 g.dm^{-3}, polysorbate 80 (Tween 80) 1 g.dm^{-3}, ammonium citrate 2 g.dm^{-3}, sodium acetate, 5 g.dm^{-3}, magnesium sulphate 0.1 g.dm^{-3}, manganese sulphate 0.05 g.dm^{-3} and dipotassium phosphate 2 g.dm^{-3}.

Growth characteristics was determined from samples of lactobacilli cultivated in MRS medium at 37 °C during 40 hours, which was carried out with constant stirring (100 rpm). Initial optical density (λ = 600 nm) of lactobacilli in broth was 0.4 and inoculum was prepared by propagation of cells in MRS broth for 24 hours at 37°C.

Characterization of growth

For analysis, samples were taken every 4 hours and growth was studied by measuring optical density (OD) at λ = 600 nm photometrically (Genesys 10S UV-VIS Spectrophotometer, ThermoScientific), dry cell weight (g.dm^{-3}) gravimetrically after drying of biomass to constant weight and by measuring of pH value of medium. Measured values of OD_{600}, dry cell weight and pH of medium were plotted on curves.

Aminopeptidase assays

To assay the cytoplasm aminopeptidase activities, cultures of both strain from the late exponential phase of growth were used. Cells were harvested by centrifugation at 9 000 x g per 10 minutes at 4 °C (Avanti® J-30I, Beckman Coulter, USA), washed with 0.1 M sterile Sorenson´s phosphate buffer (pH 7.0) and resuspended in the same sterile buffer. Cytoplasmatic extracts were prepared by ultrasonic disintegration of cells (Bandelin Sonopuls HD 3200, Germany). Smashed cells was centrifuged at 14 000 x *g* per 10 minutes at 4 °C to remove unbroken bacteria and large cellular debris. The supernatant represented crude cell-free extracts.

For determination of specific aminopeptidase activity were used substrates based on *p*-nitroanilides (*p*-Na): Phe-*p*Na, Ala-*p*Na, Leu-*p*Na, Met-*p*Na, Pro-*p*Na and Lys-*p*Na (Sigma, Germany; Serva, Germany). Activities of enzymes were determined by the method of **El Soda and Desmazeaud (1982)** based on measuring of absorbance of released *p*-nitroaniline at 410 nm. The concentration of *p*-nitroaniline was calculated by Lambert-Beer law. Results were expressed in units of activity per volume of solution (μkat.dm^{-3}).

RESULTS AND DISCUSSION

The growth of lactobacilli was studied by measuring optical density (OD) at λ = 600 nm, dry cell weight and pH value of MRS medium during cultivation in time. First of all, optical density of cell in MRS medium was measured photometrically.

Growth curve is crucial for further fermentation experiments because it shows growth phases. In industrial production are bacterial cells commonly harvested during the late exponential or stationary growth phase to ensure high cell numbers (**Laakso et al., 2011**).

Figure 1 shows growth curve of *L. plantarum* cells, where optical density was measured every 4 hours. The end of exponential phase was noticed between 24. – 28. hours of cultivation in MRS medium. Growth curve of *L. brevis* (Figure 2) shows different result, the late exponential phase was reached after 28. – 32. hours of cultivation.

The stationary phase of growth started after 28. hours *of L. plantarum* cultivation and 32. hours of *L. brevis* cultivation in MRS broth, respectively. This study describe bacterial growth phases in batch system of cultivation, where initial amount of nutrients in a MRS medium was limiting factor for the growth of lactobacilli. In the early stationary phase was observed growth retardation and stopping of cell growth. Reason is described in study by **Cohen et al. (2006)**. During growth, levels of glucose in medium are decreasing. Lack of glucose provoked induction of alternative pathways to obtain energy by carbohydrate metabolism. The energy required for induction of alternative pathways may be the reason for reducing of growth rate in late exponential and early-stationary phases.

Dry cell weight of lactobacilli was measured gravimetrically and correspond with the highest optical density of cells in MRS medium. The highest amount of dry cell weight of L. plantarum CCM 3627 was reached between 24. to 28. hours of growth and reached 0.62 $g.dm^{-3}$ of MRS broth (Figure 3). The final cell yield of L. brevis was lower, reaching a cell concentration of 0.45 g of dry

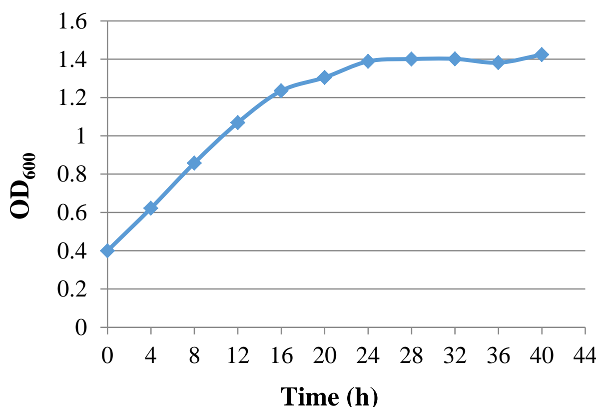

Figure 1 Growth curve of *L. plantarum* CCM 3627 during cultivation in time.

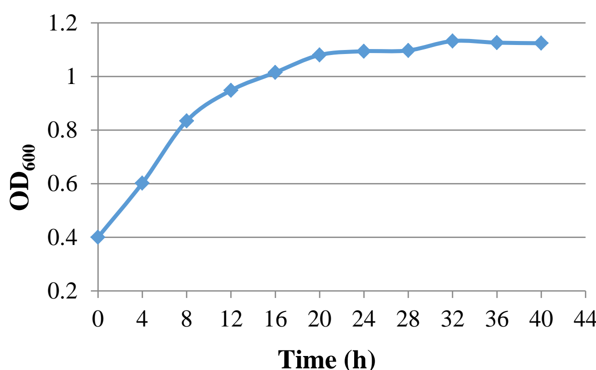

Figure 2 Growth curve of *L. brevis* CCM 1815 during cultivation in time.

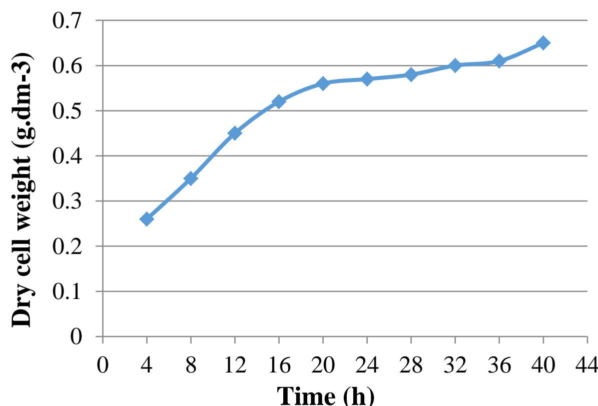

Figure 3 Increase of dry cell weight of *L. plantarum* CCM 3627 during cultivation in time.

biomass for 1 dm^3 MRS media.

One of the important technological characteristics in of lactobacilli is ability to produce organic acids and acidification of an extracellular environment. Therefore, lactobacilli are acid tolerant. Ability of acidification is important for fermentation process of cereal substrates, because low pH value of dough activate cereal proteolytic enzymes and start proteolysis of cereal proteins. Acidification also may be protection against contaminating microorganisms **(Rollán et al., 2010)**.

Measuring the pH values of the medium during cultivation showed that lactobacilli had very active carbohydrate metabolism. Significant changes of pH was observed in the first of 24 hours of cultivation time. It was produced such amount of organic acids, which decrease pH to less than 5.0 in both cases. Initial pH value of MRS medium was 6.5 and it was acidified by *Lactobacillus plantarum* to pH value less than 4.6 in the late exponential phase (Figure 5). *Lactobacillus brevis* acidify extracellular environment in exponential phase of growth to pH 5.0 (Figure 6). This is very important information for further experiments, because investigated lactobacilli have considerable potential for successful acidification of dough, which initiate proteolysis of cereal proteins.

Mechanisms of acid resistance of fermentative microorganisms are provided by the glutamate decarboxylase system, alkalization of the external environment, homoeostasis of intracellular pH, DNA and protein damage repair and changes in cell membrane. Lactobacilli are acid tolerant by production of acid shock proteins and changes in intracellular and surface-located proteins. Acid stress also induces changes in the fatty acid

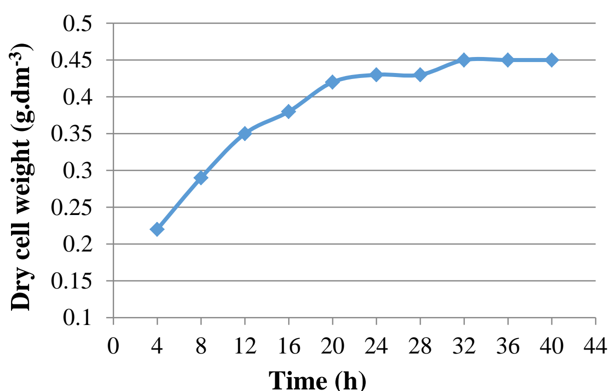

Figure 4 Increase of dry cell weight of *L. brevis* CCM 1815 during cultivation in time.

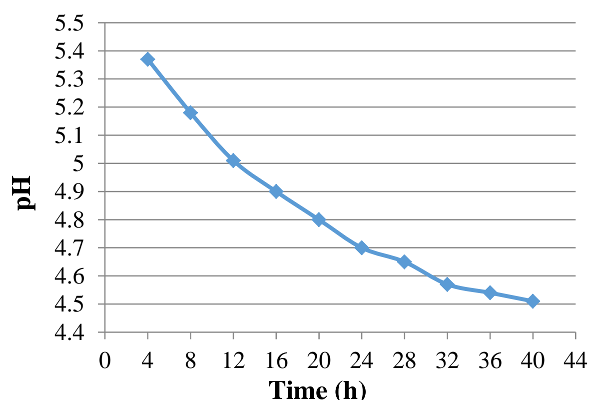

Figure 5 pH change of the MRS medium during growth of L. plantarum CCM 3627 in time.

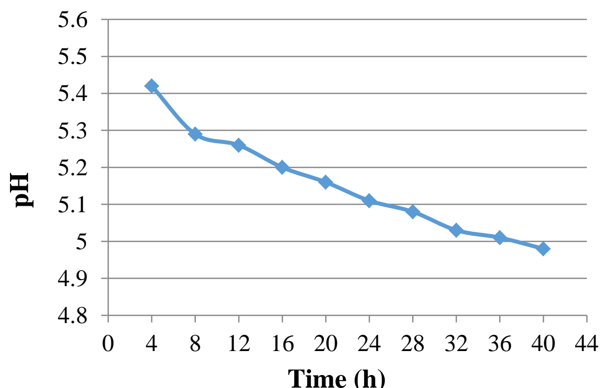

Figure 6 pH change of the MRS medium during growth of L. brevis CCM 1815 in time.

contents of the cytoplasmic membrane **(Hussain et al., 2013)**.

Laakso et al. (2011) introduced that the abundance of several stress proteins and the transcription of genes encoding stress responsive proteins was elevated when the cells reache the stationary growth phase. **Hussain at al. (2013)** added that lactobacilli are robust in their adaptive and physiological responses under different conditions. They are adaptabile because of production of the transient induction proteins and physiological changes. Their effective adaptation mechanisms enhancing the ability survive under adverse conditions. Proteins activate different metabolic pathways in stress conditions and protein profile of lactobacilli are subjected to changes.

In a experiment, the crude cell-free enzymatic extracts of both strains of *Lactobacillus* were assayed by synthetic substrates: Phe-*p*Na, Ala-*p*Na, Leu-*p*Na, Met-*p*Na, Lys-*p*Na and Pro-*p*Na. Table 1 summarizes the results obtained after hydrolytic reactions. Results were similar in both cases and were expressed in units of activity per volume of solution (µkat.dm^{-3}). *L. plantarum* CCM 3627 possessed the highest activity of lysin-specific peptidase (731 µkat.dm^{-3}), while proline-specific peptidase showed the lowest activity (69 µkat.dm^{-3}). Peptidase complex of *L. brevis* CCM 1815 reached similar values. Lysine-specific aminopeptidase reached the highest activity (410 µkat.dm^{-3}). In contrast, proline aminopeptidase activity was quite low (12 µkat.dm^{-3}). From the point of view to produce fermented bread with hydrolysed celiac-active peptides the essential finding was presence of active proline-specific peptidase, which is prerequisite for successful cleavage of peptide bonds with proline from celiac-active hardly cleavable peptides.

CONCLUSION

Lactobacillus plantarum CCM 3627 and *Lactobacillus brevis* CCM 1815 produce organic acids, as well as specific intracellular aminopeptidases during cultivation in MRS medium. Results confirm production of active proline aminopeptidase, which is important for cleavage of proline rich-peptides. Through ability acidify of environment to pH value to less than 5.0, cereal phytases and endogenous cereal proteases may be activated during dough fermentation. These enzymatic processes can result in improvement mineral bioavailability and degradation of proteins into the peptides. Investigated *Lactobacillus* strains have real potential for exploitation in production of gluten-low products. Sourdough fermentation with addition of selected lactobacilli may be an alternative technology for degradation of celiac-active cereal proline-rich peptides and improvement of nutritional properties of cereal products.

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