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Selection of lactic acid bacteria strain for simultaneous production of *α*- and *β*-galactosidases

ABSTRACT

Galactosidases are group of enzymes gaining increasing attention in recent times due to the fact they catalyse hydrolysis of galatose-containing oligosaccharide, which reduce digestibility of various food and feed products. Microbial production of galactosidases is the most efficient and widely used. The aim of this study was to selects strain of lactic acid bacteria which can be used as producer of both, α- and β-galactosidases, since bacteria with such properties can be used for improving digestibility of products containing soybean and milk derivatives. In preliminary selection only intracellular activity of α- and β-galactosidases was detected and Lactobacillus reuteri was selected as the most adequate producer of both enzymes. In subsequent experiments, fermentation was optimized with respect to produced intracellular activity of both enzymes using spectrophotometric enzyme assays. Enzymes were characterized with respect to their temperature and pH optima. It was revealed that the most effective factor for production of enzymes is the selection of inducer. The addition of lactose induced production of β-galactosidase, while raffinose was the most efficient inducer of α-galactosidase production. Further improvement of enzyme production was achieved by optimization of inoculum concentration.

Keywords: *α-galactosidase; β-galactosidase; Lactobacillus reuteri; fermentation; raffinose.*

1. INTRODUCTION

Term “galactosidases” comprises two groups of enzymes: *α*-galactosidases (*α*-D-galactoside galactohydrolases, E.C. 3.2.1.22) and *β*-galactosidases (*β*-D-galactoside galactohydrolases, E.C. 3.2.1.23). Purpose of both galactosidases is to catalyse hydrolysis of glycosidic bonds formed with participation of galactose hemiacetal hydroxyl group, but they differ in specificity with regard to galactose conformation (*α*- or *β*-anomers) involved in bond formation and, consequentially, glycosidic linkage type. In view of their application, *β*-galactosidases are by far more popular and already have established status in food industry, since their natural substrate is lactose. Therefore, they are used for enzymatic production of lactose-free milk products, which are in recent times food with rising market demand, due to increasing awareness of lactose intolerance in humans [1].

On the other hand, use of *α*-galactosidase is still limited to several small-scale applications due to the fact that its natural oligosaccharide substrates are raffinose, melibiose and stachyose, sugars which are less abundant in nature. Since raffinose inhibits sucrose crystallization from beet sugar syrups, important field of application of *α*-galactosidase is in sugar industry to improve crystallization and sugar yield by catalysing hydrolysis of raffinose [2]. It can also be used for modification of guar gum, natural galactomannan, in order to improve gelling properties.[3] However, the most interesting area of application with prospect to large-scale use of *α*-galactosidase is enzymatic modification of soybean products.[4-6] Majority of soluble sugar fraction of soybean are *α*-galactosidic oligosaccharides - raffinose, stachyose and verbascose. They can’t be easily digested in human guts, which usually results with flatulence after consumption of soybean products causing their reduced use in diet. Hence, there is rising interest in use of *α*-galactosidase pre-treated products because enzymatic hydrolysis of galacto-oligosaccharides may alleviate gastric distress caused by fermentation of carbohydrates in the large intestine, thereby improving the nutritional

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quality of legume-based foods such as soybean milk and cowpea meal [6,7].

Both enzymes are predominantly produced as extracellular or intracellular products of fermentative processes performed with various bacteria, yeasts or molds. The most important microbial sources of *α*- and *β*-galactosidases are given in Table 1. It can be seen that some of lactic acid bacteria, besides presumed *β*-galactosidases, also produce *α*-galactosidases.

Table 1 - Microbial sources of α- and β-galactosidases

α-galactosidase	**β-galactosidase**
BACTERIA	
Bacillus licheniformis *Bacillus megaterium* *Lactobacillus plantarum* *Lactobacillus reuteri* *Lactobacillus helveticus* *Lactobacillus fermentum* *Lactobacillus acidophilus* *Lactococcus lactis* *Leuconostoc mesenteriodes* *Bifidobacterium breve* *Bifidobacterium longum* *Streptomyces erythrus* *Streptomyces griseoloalbus* *Sulfolobus solfataricus*	*Bacillus circulans* *Bacillus megaterium* *Bacillus stearothermophilus* *Bacillus licheniformis* *Lactobacillus sakei* *Lactobacillus plantarum* *Lactobacillus reuteri* *Lactobacillus acidophilus* *Lactobacillus pentosus* *Lactobacillus helveticus* *Lactobacillus bulgaricus* *Lactobacillus fermentum* *Bifidobacterium infantis* *Bifidobacterium longum* *Bifidobacterium bifidum* *Pyrococcus furiosus* *Streptococcus thermophilus* *Sulfolobus solfataricus*
YEASTS	
Candida javanica *Candida guilliermondii* *Debaryomyces hansenii* *Saccharomyces cerevisiae* *Gibberella fujikuroi*	*Kluyveromyces lactis* *Kluyveromyces marxianus* *Kluyveromyces fragilis* *Cryptococcus laurentii*
FUNGUS	
Aspergillus flavipes *Aspergillus oryzae* *Aspergillus ficuum* *Aspergillus nidulans* *Penicillium chrysogenum* *Thermomyces lanuginosus*	*Aspergillus aculeatus* *Aspergillus oryzae* *Aspergillus niger* *Thermomyces lanuginosus*

This type of versatility is of great importance since microbes with such properties can be used for obtaining added-value products (with reduced concentration of flatulent oligosaccharides) from blends of soybean products (meal or flour) and cheese whey, which are currently used as animal feed. Intracellular enzymatic activities can be utilized in two ways: directly in fermentation or indirectly, as source of intracellular enzymes. Lactic acid bacteria are very favorable choice due to their GRAS ("generally recognized as safe") status in food and health products, vast knowledge of ecological factors affecting their growth and well-established procedures for their cultivation [1]. Regarding their cultivation, various growth media can be applied for lactic acid bacteria, but due to the fact galactosidases are inducible enzymes medium must contain adequate inducer molecules for achieving high enzymatic activity. Therefore, if natural complex growth medium does not contain galactose-containing oligosaccharide it must be enriched with lactose (if target is high *β*-galactosidase activity) or raffinose/melibiose (if target is high *α*-galactosidase activity).

In our study, preliminary set of experiments is designed for selection of species from our lactic acid bacterium collection that provides high activity of both, *α*- and *β*-galactosidases. Optimization of growth media for production of these enzymes will be performed and obtained enzymes will be characterized with respect to their pH and temperature optima.

2. MATERIALS

Six lactic acid bacteria (LAB) strains (*Lactobacillus acidophilus* ATCC 4356, *Lb. rhamnosus* ATCC 7469, *Lb. reuteri* ATCC 23271, *Lb. Helveticus* ATCC 15009, *Lb. delbrueckii* subsp. *Bulgaricus* ATCC 11842, *Streptococcus termophilus* S3) from the collection of the Department of Biochemical Engineering and Biotechnology (Faculty of Technology and Metallurgy, Belgrade) were screened for *α*- and *β*-galactosidase activity. The chemicals used for cultivation media preparation were obtained from Torlak Institute of Immunology and Virology (Belgrade, Serbia), while other chemicals were of analytical grade and purchased from Sigma Aldrich (St. Louis, USA) unless stated otherwise.

2.1. Microorganisms and fermentation

Lactic acid bacteria strains were stored in vials containing MRS broth medium and 50% (v/v) of glycerol at -20 ^{o}C, except in case of *S. termophilus* that was maintained in M17 broth containing glycerol under the same conditions. The culture propagation, prior to inoculation of fermentation media, was conducted three times consecutively in MRS or M17 broth, depending of the strain, under microaerophilic conditions at 37 ^{o}C.

All fermentations were performed at 37 ^{o}C in conical Erlenmeyer flasks on the rotary shaker (IKA® KS 4000i control, Werke GmbH and Co.) set at 150 rpm. Preliminary fermentations were perfor-

med as using 200 ml of fermentation media (MRS/M17 broth) under microaerophilic conditions for 24h.

2.2. Extraction of intracellular enzymes

In order to obtain galactosidases, microbial cells were harvested by centrifugation (Sigma® 2-16, SciQuip Ltd, Shropshire, UK) at 12000 rpm for 10 min. After being washed twice with 0.1M sodium phosphate buffer (pH 6.8), they were re-suspended in the same buffer. The cell suspension was there after subjected to vigorous vortexing in presence of quartz sand (Kopovi Ub, Serbia) in order to achieve cell disruption [36]. Activity of released enzyme activity was measured after removing cell debris by centrifugation (12000 rpm for 10 min).

2.3. β-Galactosidase activity assay

β-Galactosidase activity was determined using 10 mM *o*-nitrophenol-*β*-D-galactopyranoside (*o*-NPG) in 0.1 M sodium phosphate buffer (pH 6.8) as a substrate. The reaction course was monitored for 2 minutes, by measuring the concentration of released *o*-nitrophenol (*o*-NP) spectrophotometrically at 410 nm. One unit (IU) is defined as the amount of the enzyme that catalyzes the liberation of 1 μmol *o*-NP per min under the specified assay conditions. The molar extinction coefficient for *o*-NP was found to be 1357 dm^3/mol·cm.

2.4. α-Galactosidase activity assay

α-Galactosidase activity was measured using 10 mM raffinose in 0.1 M sodium phosphate buffer (pH 6.8) as a substrate. The reaction course was monitored for 2 minutes, by measuring the concentration of released galactose according to the DNS method.[47] The molar extinction coefficient for galactose was found to be 1400 dm^3/mol·cm.

2.5. Protein determination

The amount of proteins in the crude cell-free extracts was determined by Bradford method using bovine serum albumin as the standard [48].

2.6. Optimization of fermentation parameters

Lactobacillus reuteri, chosen as the best producer of both enzymes, was therefore used for further optimization of process parameters. In view of defining optimal fermentation period, fermentation was performed under previously defined conditions, and samples for monitoring bacterial growth and enzymes production were taken and analyzed during four day period.

In next set of experiments, modifications of fermentation medium (MRS broth) were introduced in order to access the best carbon sources for galactosidases production. Different carbon sources (overall concentration of 2%), namely glucose, lactose, raffinose, melibiose and their mixtures were examined. Finally, the optimal concentration of the best carbon source, for both enzymes, was determined. The range of the sugar concentration (1-4 %) was chosen on the basis of literature survey.

2.7. Effects of temperature and pH on enzymatic activity

The effect of temperature on galactosidases activity was examined by incubation of the enzyme and the substrates at various temperatures in range 30 to 60 °C. The enzyme activity measurement was conducted described earlier, and

The effect of pH was determined on optimal temperature for each enzyme by varying buffers used for substrate preparation. Sodium acetate buffer (pH 4-5.5) and sodium phosphate buffer (pH 6-8) were used for this purpose.

3. RESULTS AND DISCUSSION

3.1. Screening for α- and β-galactosidase producing microorganisms

The present work is focused on assessing the capability of several lactic acid bacteria strains to produce high levels of α-galactosidase and *β*-galatosidase activity (Table 2). Fermentation was, thus, carried out in a shake flask on a rotary shaker (150 rpm), using MRS/M17 broth as a fermentation medium at 37 °C for 24 h, as described above. After extracting intracellular activity, under conditions optimized in our previous study [36], crude cell extracts from different sources were compared from the aspect of obtained activities for both enzymes. Moreover, it should be mentioned here that no extracellular activity was detected throughout this experiment.

Table 2 - Production of α- and β-galactosidases using LAB

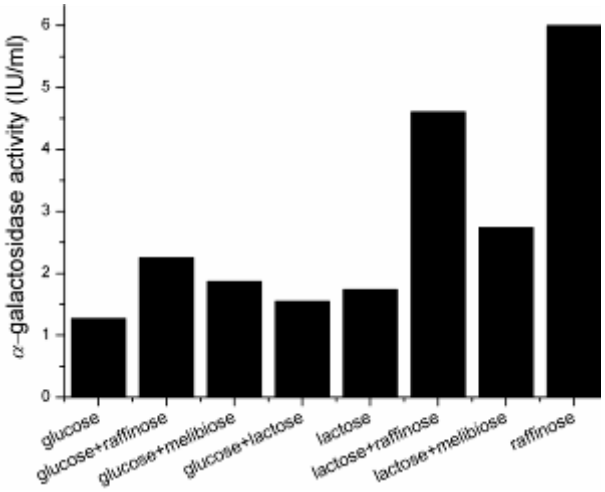
Lactic acid bacteria	***α*-galactosidase activity (IU/ml)**	***β*-galactosidase activity (IU/ml)**
Lactobacillus reuteri	1.274	0.420
Lactobacillus acidophilus	0.255	0.671
Lactobacillus rhamnosus	-	-
Lactobacillus helveticus	-	0.344
Lactobacillus bulgaricus	-	0.011
Lactobacillus delbrueckii ssp. lactis	-	-
Streptococcus termophilus	0.181	0.110

The results have shown that in terms of α-galactosidase activity, enzyme extracted from *L. reuteri* was the most active one (1.27 IU/mL), while notably less active α-galactosidases were extracted from *L. acidophilus* (0.255 IU/ml) and *S. thermophilus* (0.181 IU/ml). All the other used *Lactobacillus* strains produced negligible levels of α-galactosidase activity (Table 2). On the other hand, in case of *β*-galactosidase, highest obtained activity was extracted from *L. acidophilus* (0.671 IU/ml). Afterwards, *L. reuteri* (0.420 IU/ml) and *L. helveticus* (0.371 IU/ml) *β*-galactosidases showed lower, but still significant activities. While, rather poor activity was expressed by enzyme extracted from *S. termophilus* (0.110 IU/ml), other lactic acid bacteria strains were not capable of producing *β*-galactosidase. Therefore, according to highest obtained α-galactosidase active enzyme, and considerably high *β*-galactosidase activity, *L. reuteri* was elected as the most suitable producer of both enzymes. In order to increase efficiency of α- and *β*-galactosidase production in terms of activity of extracted enzyme, key parameters of enzyme production (such as carbon sources and inoculum size) using *L. reuteri* were optimized.

3.2. Optimization of carbon sources

It is very well documented in the literature that the highest influence on enzyme synthesis lies in the optimization of the fermentation media. There are numerous reports on significant effects of utilized carbon sources of galactosidases [16,49,50]. However, these findings are not universal, and depend greatly on the nature of producer. The first objective was to investigate the enhancement of *α*- and *β*-galacosidase activity in *L. reuteri* by varying different carbon sources in fermentation media, since both enzymes are highly inducible. Selected microbial culture was, therefore, grown separately on several modified MRS mediums containing different carbon sources (glucose, mixture of glucose and raffinose, mixture of glucose and melobiose, lactose, mixture of lactose and raffinose, mixture of lactose and melibiose, raffinose and finally, melibiose) to reach final concentration of 20 g/l.

Based on the results presented on Fig. 1A, it can be seen that *L. reuteri* cultivated on all three MRS mediums containing raffinose displayed substantial *α*-galacosidase activity production. Modified MRS medium with raffinose as sole carbon source proved to be the inducer of the maximum α-galactosidase activity production (6.00 IU/ml), while media containing raffinose mixtures with glucose and lactose displayed significant levels of α-galactosidase activity induction (2.25 IU/ml and 4.61 IU/ml, respectively). By adding glucose as sole carbon source to MRS medium, 2-folds lower α-galactosidase activity were detected comparing to MRS medium containing raffinose mixture with glucose. These results could be explained by the facts that α-galactosidase is raffinose inducible enzyme (because of gal operon stimulation [16] and, also, is subjected to catabolite repression by glucose. Melibiose is also inducer of α-galactosidase production [17], but because of the intracellular accumulation of its degradation by-product (glucose), melobiose does not have the same efficiency toward α-galactosidase production as raffinose. Previous studies also reported high levels of *α*-galactosidase activities from various lactic acid strains on raffinose [15, 16, 48].



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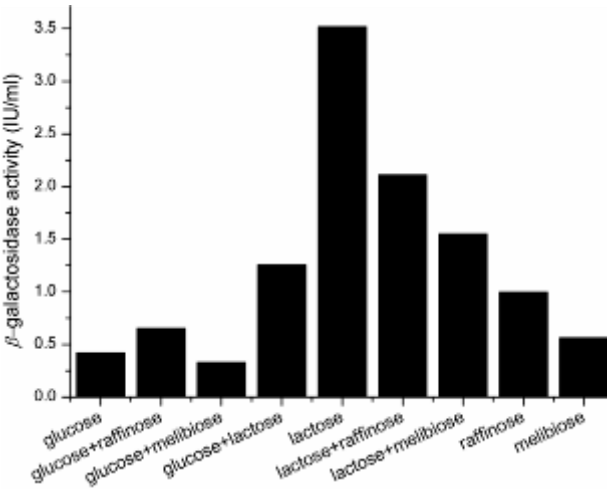
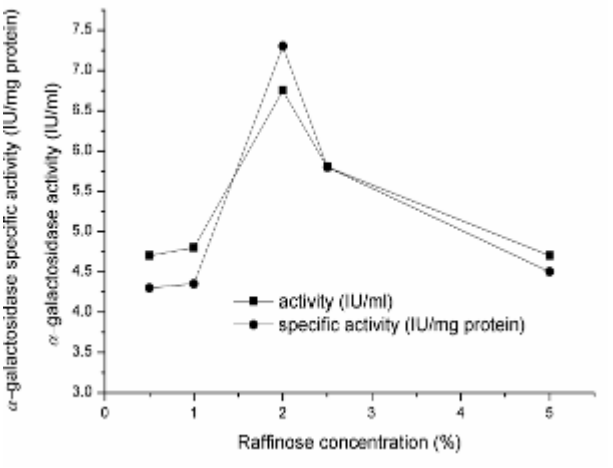


B)

Figure 1 - Effect of different carbon sources on α-galactosidase production (A) and β-galactosidase production (B)

Likewise, influence of carbon sources on obtained *β*-galactosidase activity is highly pronounced. Similarly to the raffinose induction in the previous case, herein lactose, as a primary natural *β*-galactosidase substrate, showed a great impact on produced activity (Fig. 1B). Consequently, the

highest obtained activity was extracted from the culture grown on lactose as a sole carbon source (3.52 IU/ml). At the same time, all the other mixtures containing lactose (glucose, raffinose and melibiose mixture) showed moderately high activity yields, namely 1.25 IU/ml, 2.11 IU/ml and 1.55 IU/ml, respectively. The glucose once more proved to be a worst choice in terms of produced activity, due to abovementioned catabolic repression. This hypothesis is supported with lower levels of produced activity in media containing melibiose in comparison to media containing raffinose, owing to the fact that during melibiose consumption, free glucose molecules able to act as repressor of *β*-galactosidase production. These results are in good correlation with existing literature data. There are several reports on enhancement of *β*-galactosidase production caused by lactose induction [36,49].



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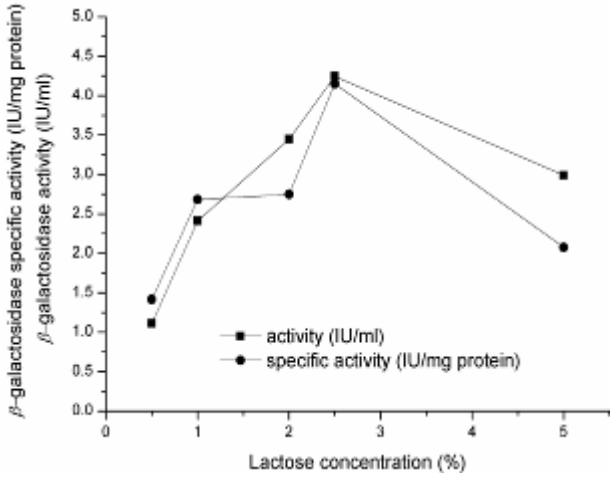


B)

Figure 2 - Effect of carbon source concentration on α-galactosidase production (A) and β-galactosidase production (B)

Besides the nature of carbon sources in the culture medium, its amount demonstrated to be equally important from the aspect of the efficient α-galactosidase production. Since raffinose as carbon source was the best inducer of α-galactosidase activity production, in further set of experiments, the concentration of raffinose in fermentation media was optimized. Different amounts of raffinose (in range of 0.5-5 % (w/v)) were used instead of glucose as substrate for α-galactosidase production in MRS medium. The activity (IU/ml) and specific activity (IU/mg proteins) of α-galactosidase increased progressively with increase of initial raffinose concentrations up to 2 % (Fig. 2A), while further growth of initial raffinose concentration led to reduction in both activity and specific activity of α-galactosidase. This trend implies that together with an enhancement of raffinose content, there is an increase of degradation products content which leads to repression of inducible *β*-galactosidase production. It can be stated that the efficient α-galactosidase production depends on a balance between raffinose induction and intracellular galactose or saccharose repression. However, Yoon and his co-workers found that maximum activity production by *L. curvatus* was achieved in modified MRS medium with raffinose concentration of 1 %, although the decrease was more lenient than in our experiment. This just finding confirms the fact that not every strain has the same resistance toward the concentration of repressors.

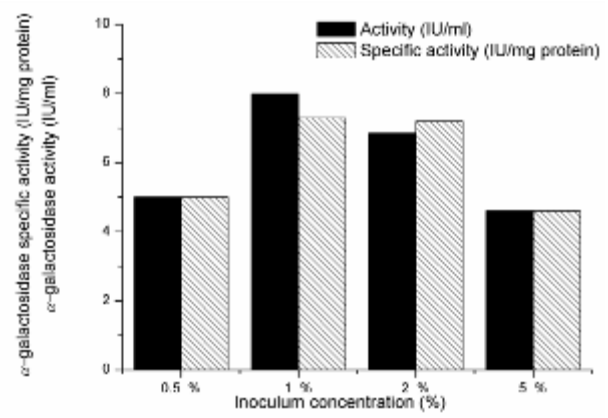
In case of *β*-galactosidase production, similar results were obtained. However, both activity (IU/ml) and specific activity (IU/mg protein) reach the maximum values when 2.5 % lactose concentration is employed (Fig. 2B). Further increase of lactose, would cause increase of intracellular glucose content, which subsequently leads to *β*-galactosidase repression. These results are similar to those previously obtained by the same group of authors for *L. acidophilus*. Anyhow, Murad and coworkers proved that the highest obtained *β*-galactosidase activity (2.54 IU/ml) was accomplished at 3-folds higher lactose content [51].

3.3. Optimization of inoculum concentration

After determination of optimal raffinose concentration, in further set of experiments, the influence of initial inoculum concentration on activity and specific activity of produced α-galactosidase was investigated. Four different amounts of inoculum 0.5 %, 1 %, 2.5 % and 5 % (v/v) were added to the MRS medium containing 2 % of raffinose to initiate fermentation process. The obtained results, presented on Fig. 3, showed that α-galactosidase exhibited the highest activity (8 IU/ml) and specific activity (7.2 IU/mg protein) at inoculum concentration of 1 % (v/v).

Similarly, at inoculum concentration of 1 % *β*-galactosidase, as well, exhibited the highest activity (4.57 IU/ml) and specific activity (4.36 IU/mg protein). In both cases, specific activities obtained using 1 % and 2 % inoculum concentration are

insignificantly different, yet higher expressed activities were different, thus we chose inoculum concentration of 1 % as optimal value of this parameter. These results confirm previously imposed statement that an increase of degradation product content leads to repression of galactosidase production, since higher inoculum concentration corresponds to higher substrate consumption, an eventually increase od degradation products.



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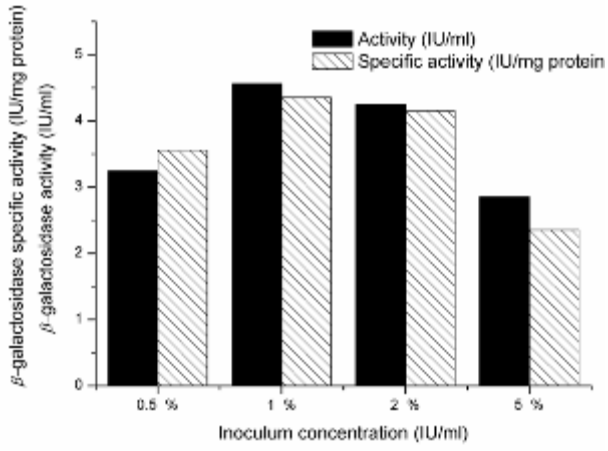


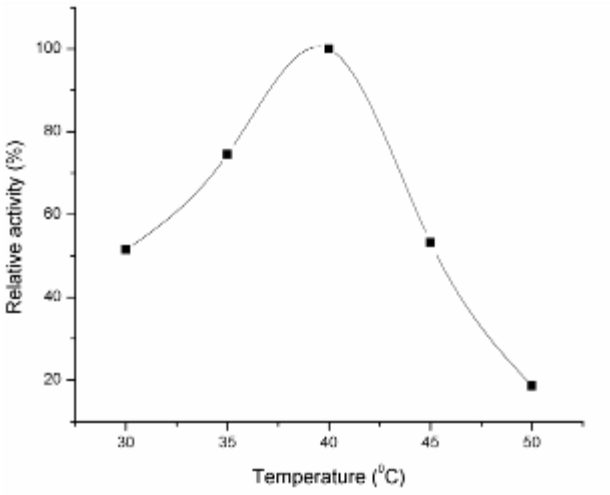
B)

Figure 3 - Effect of inoculum concentration on α-galactosidase production (A) and β-galactosidase production (B)

3.4. Temperature and pH

Enzyme characteristics vary greatly in view of the nature of their producer. For example, the vast majority of lactic acid bacteria enzymes are mesophilic enzymes active on ambient temperatures, but this can be subjected to change depending on the strain.

The effect of temperature on *α*- and *β*-galactosidase activity was examined by incubating enzymes and their substrates at appropriate temperatures (Fig.4). The obtained result showed that *α*-galactosidase activity increased with the increase of temperature and the maximum *α*-galactosidase activity was found at 40 °C. Afterwards, activity rapidly decreased, most likely due to the thermal inactivation. Similar temperatures were reported for *α*-galactosidase from *L. helveticus* (37 °C) [12] and *L. acidophilus* (37 °C) [10], while higher temperatures were reported for *L.fermentum* (50 °C) [11,52] and *L. plantarum* (45-50 °C)[9]. On the other hand *β*-galactosidase showed broad range of optimal temperatures 40-50 °C. These results match previously reported results for *L. Reuteri* [53].



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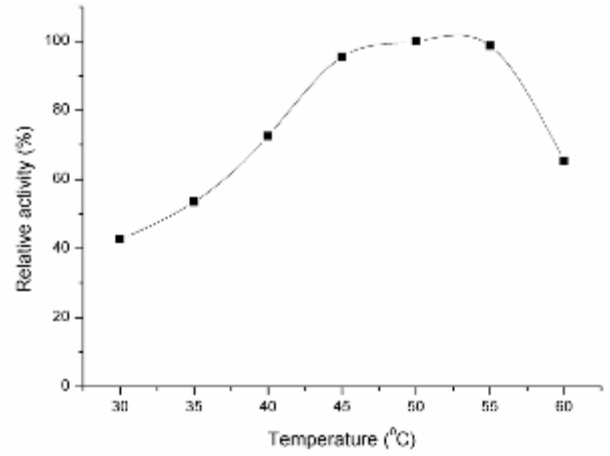
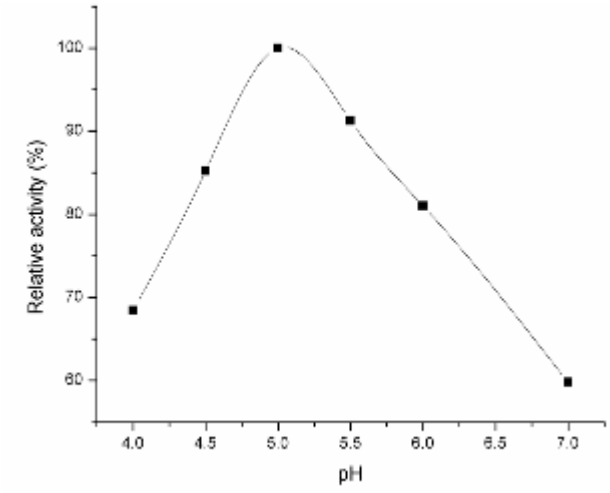


B)

Figure 4 - Effect of temperature on α-galactosidase activity (A) and β-galactosidase activity (B)

The studying of influence of pH on enzymatic activity of *α*-galactosidase is depicted in Fig. 5A. As it can be seen from the figure, the maximum activity for obtained *α*-galactosidase from *L. reuteri* was observed at pH 5.0. The activity of enzyme dramatically decreased at neutral pH values. Similar results have been reported by other authors [13]. Nevertheless, *β*-galactosidase dependence on pH value showed different trend. Optimal values were shifted towards more neutral pH (around pH 7) values, similarly to previously reported data concerning lactic acid bacteria *β*-galactosidases [54].



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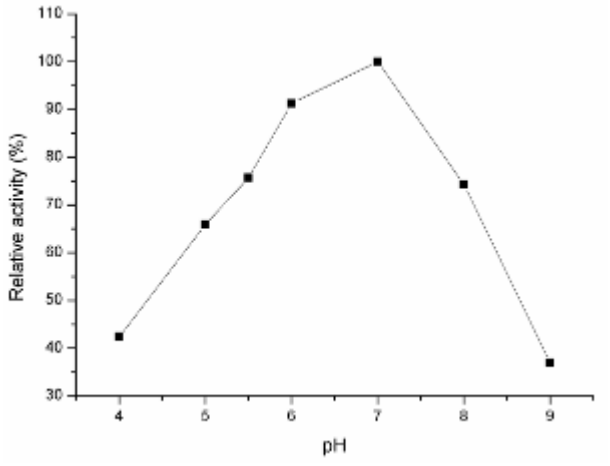

B)

Figure 5 - Effect of pH on α-galactosidase activity (A) and β-galactosidase activity (B)

4. CONCLUSION

In this study was clearly shown that some strains of lactic acid bacteria are simultaneous producers of both, *α*-and *β*-galactosidases. Among them *Lactobacillus reuteri* was the most potent producer and optimization of fermentation with respect to produced intracellular enzymatic activity revealed that addition of adequate inducers can be used as control of produced enzymatic activity. Significant product activities of both galactosidases and probiotic properties of *L. reuteri*, render this microorganism as very interesting for solid state fermentation and improvement of digestion of feed products containing soybean products and whey.

Acknowledgement

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IZVOD

IZBOR MLEČNOKISELINSKE BAKTERIJE ZA ISTOVREMENU PROIZVODNJU α– I β–GALAKTOZIDAZA

Galaktozidaze obuhvataju grupu enzima koji poslednjih godina privlače sve veću pažnju zahvaljujući činjenici da katalizuju reakciju hidrolize različitih galakto-oligosaharida prisutnih u širokom spektru proizvoda za prehrambenu i stočnu industriju, čime omogućavaju njihovu bolju svarljivost i iskoristivost. Najefikasnije i najčešće korišćene galaktozidaze vode poreklo iz mikrobnih izvora. Cilj ove studije bio je odabir soja bakterije mlečne kiseline koja se može koristiti za proizvodnju α- i β-galaktozidaza, imajući u vidu da se ove bakterije bez ograničenja mogu koristiti za poboljšavanje svarljivosti proizvoda na bazi soje i mleka. U preliminarnim eksperimentima detektovana je intracelularna aktivnost α- i β-galaktozidaza, a bakterija Lactobacillus reuteri je izabrana kao najadekvatniji proizvođač oba enzima. U narednim eksperimentima, fermentacija je optimizovana u clju poboljšanja proizvodnje intracelularnih enzima, praćenjem aktivnosti primenom spektrofotometrijskih enzimskih testova. Enzimi su potom okarakterisani sa aspekta optimalnih vrednosti temperature i pH za njihovu maksimalnu hidrolitičku aktivnost. Otkriveno je da najvažniji faktor za proizvodnju enzima predstavlja izbor induktora. Naime, dodavanje laktoze u fermentacionu podlogu dovelo je da indukuje proizvodnju β-galaktozidaze, dok se rafinoza pokazala kao najefikasniji induktor proizvodnje α-galaktozidaza. Dalje unapređenje proizvodnje ovih enzima postignuto je optimizacijom koncentracije inokuluma.

Ključne reči: *α-galaktozidaza; β-galaktozidaza; Lactobacilus reuteri; fermentacija; rafinoza.*

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