Electronic Journal of Biotechnology 17 (2014) 204–210

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Prolyl endopeptidase — Optimization of medium and culture conditions for enhanced production by *Lactobacillus acidophilus*

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ARTICLE INFO

Article history:
Received 28 February 2014
Accepted 9 July 2014
Available online 30 July 2014

Keywords:
Central composite design
Plackett–Burman design
Response surface methodology

ABSTRACT

Background: Lactic acid bacteria are able to reduce the immunoreactivity of proteins of cereal grains during wheat dough fermentation or may be a source of proteolytic preparations added during bread making. The key enzyme in prolamin degradation is prolyl endopeptidase. This study was aimed at optimizing the composition of a culture medium and culture conditions that would enhance the synthesis of intracellular prolyl endopeptidase (PEP) by *Lactobacillus acidophilus* 5e2.

Results: The application of Plackett–Burman screening plans enabled demonstrating that the concentration of a nitrogen source in the culture and the initial pH value of the culture medium were significant for PEP synthesis. Further optimization conducted with the method of central composite designs (CCD) confirmed both the linear and square impact of nitrogen concentration and initial pH value of the culture medium on PEP production. In turn, the response surface method (RSM) allowed determining the optimal nitrogen concentration and pH value at 26.88 g/l and pH 4.85, respectively.

Conclusions: Validation of the resultant model enabled over 3-fold increase in the quantity of the synthesized enzyme.

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1. Introduction

Prolyl endopeptidase (PEP, EC 3.4.21.26) is a proteolytic enzyme that hydrolyzes peptide bonds formed by proline residues located outside the polypeptide chains [1]. Proline – being a cyclic amino acid – serves a significant physiological function in preventing the enzymatic degradation of proteins. It occurs in biologically-active peptides that take part in the pathogenesis of depression, Parkinson disease or celiac disease [2,3,4].

Celiac disease is a chronic inherent disease of the small intestine affecting both children and adults with an incidence rate of 1:100–1:300 [5]. Its pathogenesis is strictly linked with systemic disorders and consumed foods, and specifically with storage proteins of cereal grains. The development of celiac disease is also predisposed genetically, with its markers including antigens of the major histocompatibility complex (MHC) class II — HLA-DQ2 and/or HLA-DQ8, occurring in respectively ca. 90–95% and 5–10% of the patients [6,7]. Factors implied in celiac disease induction include glutamine- and proline-rich peptides released from storage proteins of cereals. The presence of proline in peptides makes them resistant to digestion by enzymes of the gastrointestinal tract of man [8].

Many authors point to the feasibility of the effective degradation of immunoreactive peptides released from wheat gliadins with the use of microbial prolyl endopeptidase [1,7,8]. This enzyme is synthesized in human tissues, however its activity was not confirmed in the gastrointestinal tract nor in the intestinal brush border membrane, which may be the reason behind difficulties in digestion of proline-rich peptides [1].

So far prolyl endopeptidase has been successfully isolated from *Myxococcus xanthus*, *Flavobacterium menigosepticum*, and *Sphingomonas capsulata* bacteria [9]. The use of these enzymes for gluten degradation in the human gastrointestinal tract is, however, restricted owing to their properties [10]. They are released to the cytoplasmic membrane and are not secreted outside a cell. They reach their optimal activity at a pH value closed to neutral. A typical trait of prolyl endopeptidase preparations produced from these bacteria is the presence of unique domains blocking high-molecular peptide access to the active center [11].

Other microorganisms that synthesize proline-specific proteases include *Aspergillus niger* molds and lactic acid bacteria [10,12,13,14]. Prolyl endopeptidase isolated from a culture of *A. niger* is capable of hydrolyzing not only oligopeptides but also proteins. It exhibits optimal activity in the medium with acidity of pH 4–5 and is resistant to digestion with pepsin [10].

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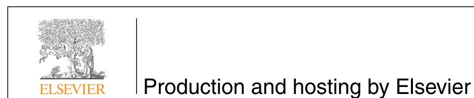
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.



http://dx.doi.org/10.1016/j.ejbt.2014.07.003
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Contrary to mold fungi, lactic acid bacteria may be used to reduce the immunoreactivity of proteins of cereal grains during wheat dough fermentation or may be a source of proteolytic preparations added during bread making [13]. Some strains of lactic acid bacteria exhibit the proline-specific activity of both endopeptidase and endoprotease [12,13]. They include *Lactobacillus acidophilus* 5e2 strain which displays the activity of proline endopeptidase reaching 25.48 U/mg protein [13].

The aim of this study was to optimize the composition of a culture medium and conditions of prolyl endopeptidase synthesis by *L. acidophilus* 5e2.

The optimization of culture conditions facilitates more effective consumption of culture medium components. It enables more time- and cost-effective synthesis of greater quantities of the enzyme. The optimization was conducted using the Plackett–Burman screening designs that allow eliminating both physical and chemical factors which have no effect on the biosynthesis of prolyl endopeptidase. Statistically significant values of PEP biosynthesis conditions and culture medium composition achieved with this method constituted initial data for process optimization with the method of central composite designs. The aforementioned methods of optimization were successfully applied to determine statistically significant parameters of proteolytic enzymes synthesis by bacteria and filamentous fungi [15,16].

2. Materials and methods

2.1. Materials

Glucose, lactose, malt extract, soymeal peptone, K_2HPO_4, $(NH_4)_2C_6H_6O_7$, CH_3COONa, $MgSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$ were from Merck (Darmstatd, Germany) whereas vegetable peptone, Tween 80 and *Z*-Gly-Pro-*p*Na were from Sigma-Aldrich (St. Louis, MO, USA). The remaining reagents were purchased at POCH (Gliwice, Poland).

2.2. Microorganism

The study was conducted with lactic acid bacteria strain *L. acidophilus* 5e2 (Danisco Biolacta, Olsztyn, Poland), stored at a temperature of -35°C on MRS-broth (Merck, Darmstatd, Germany) with the addition of 25% (v/v) glycerol. In order to prepare the inoculum, the strain was double-passaged at 37°C for 12 h each time transferring 1% (v/v) of the culture onto the MRS-broth.

2.3. Prolyl endopeptidase assay

A solution (0.1 ml) containing prolyl endopeptidase was suspended in a phosphate buffer acc. to Sörensen (2.85 ml, 66 mM, pH 7.0) and substrate *Z*-Gly-Pro-*p*Na (50 μl, 20 mM in methanol) were added. The reaction mixture was incubated for 15 min at a temperature of 37°C under constant stirring. The reaction was arrested by the addition of 0.5 ml of acetic acid (30%, v/v, 7°C). The precipitate was removed by centrifugation (8000 g, 10 min, 21°C) and then the absorbance of the sample was measured at the wavelength of 410 nm (Spectrophotometer DU-650, Beckman Coulter, Fullerton, CA, USA). The PEP activity unit was expressed as 1 μmol of *p*-nitro-aniline (*p*Na) released within 1 min at 37°C and pH 7.0. The specific activity of PEP was expressed by the number of activity units per 1 mg of protein.

2.4. Determination of protein content

The content of protein was determined with the Lowry's method in Peterson's modification using a Total Protein Kit (TP0300, Sigma-Aldrich, St. Louis, MO, USA) according to the producer's procedure.

2.5. Culture conditions

A modified culture medium was used in the study, which was based on the composition of a standard MRS-broth. This modification consisted in a change of carbon and nitrogen sources. The composition and concentration of the other medium components remained unchanged. In the modified MRS-broth, lactose, glucose or malt extract served as the source of carbon, whereas soymeal peptone or vegetable peptone served as the source of nitrogen. All components were added in doses of 20 and 22 g/l of the medium in the case of, respectively, carbon and nitrogen sources.

Having been autoclaved (15 min, 121°C), the modified MRS-broth (90 ml) was inoculated with a 12-h culture of *L. acidophilus* 5e2 bacteria (10 ml). The culture was run in Erlenmeyer flasks (100 ml) without aeration, at 37°C for 15 h. Next, bacterial biomass was separated from the culture by centrifugation (5000 g, 20 min, 5°C), and then enzymes were isolated from the biomass. The resultant preparations were determined for PEP activity and protein content.

2.6. Intracellular protein extraction

The bacterial biomass was double-rinsed with a sterile physiological solution (20 ml, 8.5 g/l NaCl), and each time separated by centrifugation (5000 g, 20 min, 5°C). A suspension of biomass in a phosphate buffer (100 g/l) was prepared acc. to Sörensen (66 mM, pH 7.0) and subjected to ultrasound disintegration (pulse on: 4 s, pulse off: 1 s, 5°C, 30 min, VCX500 Vibre Cell, Sonics, Newtown, CT, USA). The resultant homogenate was centrifuged (5000 g, 15 min, 5°C) to separate the damaged cells. The supernatant containing intracellular enzymes was used for further analyses.

2.7. Experimental design and statistical analyses

All statistical analyses were carried out using Statistica software (version 10, StatSoft Inc., Tulsa, OK, USA). At the first stage of the study, the composition of culture medium was optimized using one-way analysis of variance of analytical results. It enabled selecting medium components that facilitated PEP synthesis.

2.8. Plackett–Burman designs

The effect of culture medium composition (sources of nitrogen and carbon) and culture conditions (time, temperature, acidity) on the synthesis of prolyl endopeptidase was determined with the mathematical method of the Plackett–Burman screening designs. This method allowed identifying which of the initially-determined parameters of bacterial culture had a statistically significant effect on this enzyme synthesis. It was assumed that the biosynthesis of prolyl endopeptidase depended on seven parameters of the culture: concentrations of carbon source (X_1) and nitrogen source (X_2), temperature (X_3), initial pH value of the medium (X_4), culture time (X_5), inoculum dose (X_6), and intensity of medium stirring (X_7). These parameters were analyzed at three levels of their values (Table 1): high (+1), baseline (0) and low (-1). The baseline level (0) corresponded to central values of the screening design. Based on the Plackett–Burman design, a 2^{7-4} matrix that included 8 experiments was composed. The experiments were conducted in a random order, in two replications for the entire design and in four replications for the central points.

2.9. Central composite designs

The factors selected based on the screening design as having a significant effect on PEP synthesis were subjected to further optimization with the response surface method (RSM) using central composite designs (CCDs). For two statically significant factors, a 2^2

Table 1
Experimental variables at different levels used for the production of prolyl endopeptidase by *L. acidophilus* 5e2 using the Plackett–Burman design and for first and second step of CCD.

Variable	Units	Symbol code	Experimental values				
			Axial (-1.414)	Lower (-1)	Center (0)	Higher (+1)	Axial (+1.414)
Plackett–Burman design							
Malt extract	g/l	X_1	–	10	20	30	–
Nitrogen source[a]	g/l	X_2	–	11	22	33	–
Temperature	°C	X_3	–	30	37	45	–
Initial acidity	pH	X_4	–	5.7	6.7	7.7	–
Incubation period	h	X_5	–	10	15	20	–
Inoculum size	ml/l	X_6	–	50	100	150	–
Agitation	rpm	X_7	–	50	150	250	–
First step of CCD							
Nitrogen source[a]	g/l	X_2	6.4	11	22	33	37.6
Initial acidity	pH	X_4	5.3	5.7	6.7	7.7	8.1
Second step of CCD							
Nitrogen source[a]	g/l	$X_{2'}$	6.4	11	22	33	37.6
Initial acidity	pH	$X_{4'}$	3.6	4.0	5.0	6.0	6.4

[a] Mixture of vegetable and soymeal peptone in mass ratio 15:7.

experimental matrix was prepared with 4 center points of the design, enlarged by the center point in double replication and 4 star points. A distance of $\alpha \pm 1.414$ was kept between the star points and the center in order to maintain the orthogonal character of the model. Relations between the values of initial variables and coded levels were presented in Table 1. To determine the effect of selected factors on PEP synthesis, the experiment was conducted using a culture medium with nitrogen source concentrations varying from 11.0 to 33.0 g/l, and initial acidity ranging from pH 5.7 to pH 7.7 at center points of the design. The experimental design assumed 10 experiments that were repeated twice in a random order.

Nitrogen concentration and medium acidity at which the PEP synthesis would reach maximum values were not determined for the analyzed input data. Therefore, based on the determined regression coefficients, successive experiments were planned based on the same 2^2 matrix but with altered values of medium pH value (Table 1) at center points of the design in the range of pH 4.0 to pH 6.0. In this case, the experimental design also included 10 experiments that were repeated twice in a random order. The adequacy of mathematical model fit was estimated based on the value of a determination coefficient, whereas model adequacy was checked using an *F*-test at a significance level of 5%. Plots of surface response were applied to determine the optimal values affecting the synthesis of intracellular prolyl endopeptidase.

3. Results and discussion

3.1. Selection of carbon and nitrogen sources

Modified culture media were prepared in order to determine the impact of medium composition on prolyl endopeptidase synthesis by *L. acidophilus* 5e2. To this end, standard components of the MRS-broth, *i.e.* yeast and meat extract, peptone from casein and glucose, were replaced by lactose, malt extract, as well as vegetable and soymeal peptone. Experiments demonstrated significant differences in PEP synthesis (ANOVA, Bonferroni test, $p < 0.05$, Table 2) depending on the applied individual source of carbon and nitrogen in the culture medium. *L. acidophilus* 5e2 was shown to synthesize greater quantities of PEP (32.68 U/mg) in the medium containing malt extract and vegetable peptone. In contrast, the lowest values of PEP activity were assayed in the culture with lactose and vegetable peptone (8.64 U/mg).

Further experiments were conducted with mixtures of carbon and nitrogen sources. The malt extract and vegetable peptone were left in the medium in concentrations of 13 g/l and 15 g/l, respectively, whereas the remaining components were added in a dose of 7 g/l in order to enhance PEP synthesis. Results presented in Table 2 demonstrate a statistically significant (ANOVA, Bonferroni test, $p < 0.05$) increase in the activity of prolyl endopeptidase to 38.80 U/mg in the culture medium containing malt extract, vegetable peptone and, additionally, soymeal peptone. In bacterial cultures incubated in the other variants of culture media composition, the activity of PEP was lower than in the variant with malt extract and vegetable peptone in the medium. Similar observations were made in previous studies that proved the enhanced activity of proteolytic enzymes synthesized by microorganisms on culture media containing complex sources of carbon and nitrogen, compared to the culture media containing monosaccharides like glucose which may induce catabolite repression [15,17]. The experiments demonstrated that the culture medium containing malt extract as a source of carbon and a mixture of vegetable and soymeal peptone as a source of nitrogen had a positive effect on PEP synthesis by *L. acidophilus* 5e2 and were used in the successive studies.

3.2. Screening design

The Plackett–Burman screening design was applied to evaluate seven input variables. The experimental design of the screening of the variables affecting prolyl endopeptidase synthesis by *L. acidophilus* 5e2 was presented in Table 3. The variables for which the experimentally-determined probability value *p* was lower than 0.05 had a significant impact on the synthesis of the analyzed enzyme and were subject to optimization in the consecutive stages of the study. Based on the Student's *t*-test (ANOVA, $p < 0.05$), a

Table 2
Effect of carbon and nitrogen sources on prolyl endopeptidase activity (U/mg) synthesized by *L. acidophilus* 5e2.

Carbon source	Nitrogen source		
	Soymeal peptone (22 g/l)	Vegetable peptone (22 g/l)	Vegetable peptone (15 g/l) + soymeal peptone (7 g/l)
Glucose (20 g/l)	28.50^{c}	19.26^{e}	–
Lactose (20 g/l)	14.11^{f}	8.64^{h}	–
Malt extract (20 g/l)	18.61^{e}	32.68^{b}	38.80^{a}
Malt extract (13 g/l) + glucose (7 g/l)	–	11.75^{g}	21.94^{d}
Malt extract (13 g/l) + lactose (7 g/l)	–	15.06^{f}	27.43^{c}

Means in the table followed by different letters create a homologous group which are not significantly different (ANOVA, Bonferroni test, $p < 0.05$).

Table 3
Nine-trial Plackett-Burman design matrix for seven variables with actual values along with the observed prolyl endopeptidase activity produced by *L. acidophilus* 5e2.

Runs	Experimental values							Prolyl endopeptidase activity (U/mg)
	X_1 (g/l)	X_2 (g/l)	X_3 (°C)	X_4 (pH)	X_5 (h)	X_6 (ml/l)	X_7 (rpm)	
1	-1(10)	-1(11)	-1(30)	+1(7.7)	+1(20)	+1(150)	-1(50)	25.9
2	+1(30)	-1(11)	-1(30)	-1(5.7)	-1(10)	1(150)	+1(250)	57.7
3	-1(10)	+1(33)	-1(30)	-1(5.7)	+1(20)	-1(50)	+1(250)	73.7
4	+1(30)	+1(33)	-1(30)	+1(7.7)	-1(10)	-1(50)	-1(50)	55.7
5	-1(10)	-1(11)	+1(45)	+1(7.7)	-1(10)	-1(50)	+1(250)	22.0
6	+1(30)	-1(11)	+1(45)	-1(5.7)	+1(20)	-1(50)	-1(50)	33.5
7	-1(10)	+1(33)	+1(45)	-1(5.7)	-1(10)	1(150)	-1(50)	73.0
8	+1(30)	+1(33)	+1(45)	+1(7.7)	+1(20)	1(150)	+1(250)	41.7
9(C)	0(20)	0(22)	0(37)	0(6.7)	0(15)	0(100)	0(150)	37.0

statistically significant effect on PEP synthesis was determined in the case of two variables, *i.e.* concentration of nitrogen source ($p = 0.012$) and initial pH value of the culture medium ($p = 0.020$), (Table 4). Of all the analyzed variables, a change in the initial acidity of the culture medium had a negative effect on PEP synthesis, whereas the optimization of nitrogen source concentration turned out to positively affect the biosynthesis process. The statistically insignificant variables, *i.e.* source of carbon, time and temperature of incubation, inoculum dose and stirring intensity, were omitted in the successive stages of optimization.

3.3. Optimization by CCD

The factors selected based on the screening design as having a significant effect on PEP synthesis were subjected to further optimization. This stage of the study described the impact of the nitrogen concentration in the culture medium (X_2) and the initial acidity of the medium (X_4), on PEP production by *L. acidophilus* 5e2. In turn, values of center points were adopted for the remaining input variables: X_1, X_3, X_5, X_6 and X_7. The design matrix and experimental results achieved with the central composite design method were presented in Table 5. The analysis of variance demonstrated the significance of the investigated parameters in PEP synthesis ($p < 0.05$). The initial pH value of the culture medium and concentration of nitrogen source in the medium had a linear and a square type effect on PEP biosynthesis (Fig. 1a). In contrast, no common effect of these variables on PEP production was noted in the study. The response surface for PEP production in the function of the initial medium acidity and nitrogen concentration was depicted in Fig. 1b. The plotted surface indicated a large slope alongside the axis of X_4 variable (pH) with no maximum value of PEP synthesis. The effectiveness of PEP synthesis was observed to increase with a decreasing initial pH value of the medium. The maximum values of PEP activity were recorded at the minimal values of initial medium acidity and nitrogen compound concentration in the medium ranging from 20.0 to 30.0 g/l. The analysis of results with the surface response method suggests that the maximum values of PEP activity occurred outside the analyzed range of the values of input variables. Hence, for further optimization of culture parameters, the second stage of the study was planned based on an experimental matrix with altered values of $X_{4'}$ parameter, *i.e.* initial acidity of the culture medium.

The second stage of optimization was also based on CCD, for which a matrix was prepared with altered values of the initial acidity of the culture medium at the center point (pH 5.0), at major points (pH 4.0 and pH 6.0), and at star points (pH 3.6 and pH 6.4) (Table 2). Values at the star points were lower than in the first stage of CCD in order to shift the experimental area.

In the second experiment, the surface response for PEP production attained the maximum values in the analyzed range of input variables $X_{2'}$ and $X_{4'}$, as depicted in Fig. 1. The second order regression equation was determined based on the analysis of variance ANOVA. The Fisher test (*F*-test) characterized by a low coefficient of probability $p < 0.05$ (Table 6) indicates that the model is statistically significant. The determination coefficient of the entire model (R^2) attains the value of 0.8703 (the value > 0.75 indicates good fit of the model to experimental data). The R^2 value informs to what extent the observed variability may be explained by experimental parameters and their interactions. The R^2 coefficient determined in our study indicated that the model explained 87.03% of variability of the determined

Table 4
Estimated effect, regression coefficient and corresponding *t* and *p* values for prolyl endopeptidase activity for the seven variable Plackett–Burman designs and in second step of CCD experiment.

Variables	Effect	Coefficient	Standard error	*t*-value	*p*-value
Plackett-Burman design					
Malt extract	-1.25	-0.63	6.12	-0.20	0.848[d]
Nitrogen source[a]	26.45	13.23	6.12	4.32	0.012[e]
Temperature	-10.40	-5.20	6.12	-1.70	0.164[d]
Initial acidity	-22.85	-11.43	6.12	-3.74	0.020[f]
Incubation period	-8.20	-4.10	6.12	-1.34	0.251[d]
Inoculum size	3.40	1.70	6.12	0.56	0.608[d]
Agitation	2.00	1.00	6.12	0.33	0.760[d]
CCD					
Constant	85.40	-782.80	102.27	-7.65	0.000[e]
Nitrogen source[a] (1L[b])	29.06	13.85	2.22	6.24	0.000[e]
Nitrogen source[a] (1Q[c])	-34.33	-0.14	0.03	-4.73	0.000[f]
Medium initial acidity (2L[b])	12.02	274.54	37.22	7.38	0.000[e]
Medium initial acidity (2Q[c])	-48.18	-24.09	3.63	-6.64	0.000[f]
1L by 2L	-27.65	-1.26	0.35	-3.56	0.003[f]

[a] Mixture of vegetable and soymeal peptone in mass ratio 15:7.
[b] L: linear effect.
[c] Q: quadratic effect.
[d] Non-significant at $p < 0.05$.
[e] Significant positive effect.
[f] Significant negative effect.

Table 5
Coded experimental design and results for the response surface of prolyl endopeptidase production by *L. acidophilus* 5e2 as function of nitrogen source level and initial acidity of medium.

Runs	Variables		Prolyl endopeptidase activity (U/mg)	
	$X_2/X_{2'}$	$X_4/X_{4'}$	First step of CCD	Second step of CCD
1	-1	-1	45.2	15.2
2	-1	+1	21.8	36.2
3	+1	-1	49.5	80.4
4	+1	+1	25.5	46.1
5	-1.414	0	24.4	36.2
6	+1.414	0	43.6	65.3
7	0	-1.414	78.6	15.2
8	0	+1.414	20.6	58.6
9 (C)	0	0	58.6	85.0
10 (C)	0	0	57.4	85.8

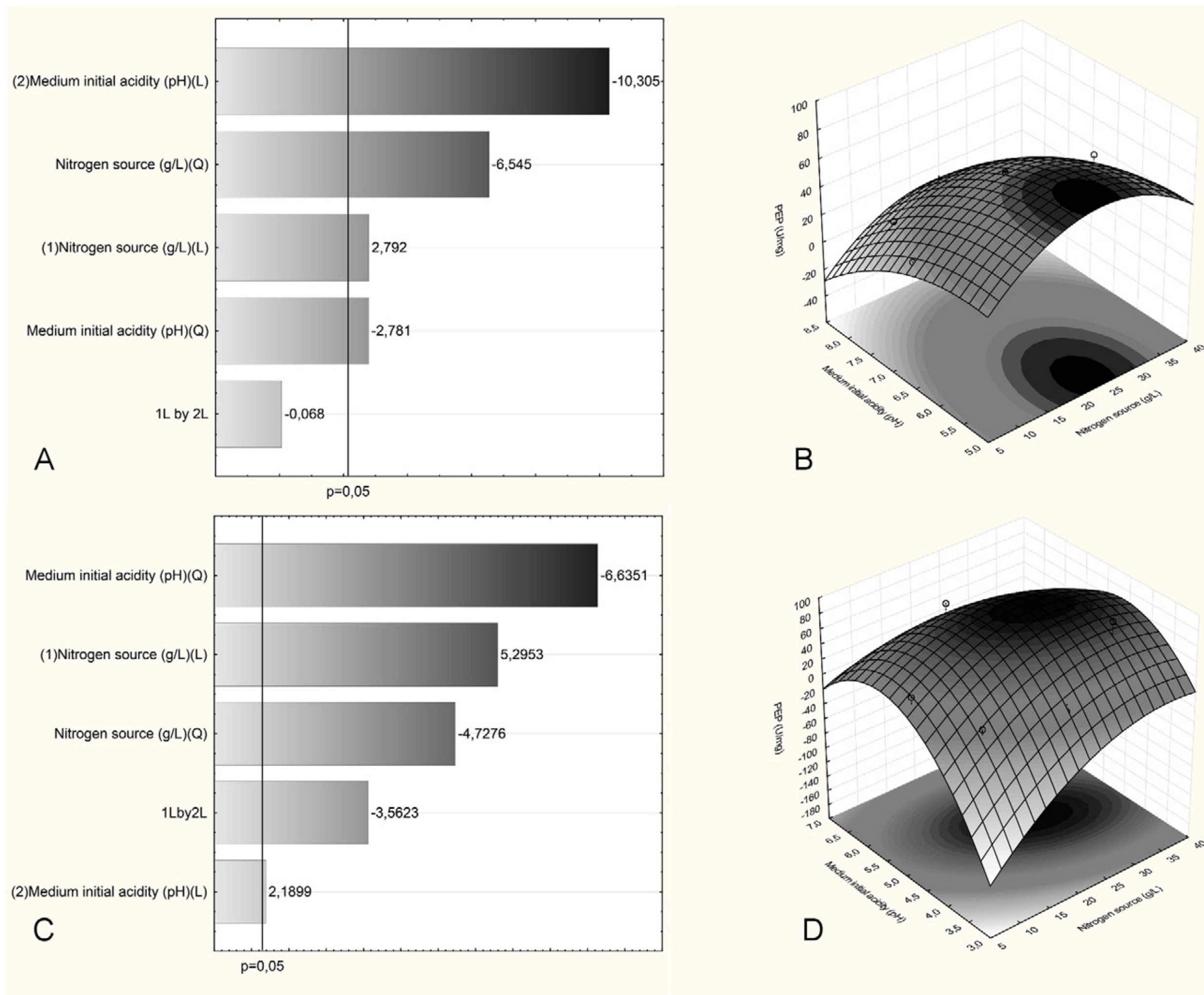

Fig. 1. Pareto chart (A, C) for the effects of variables and response surface (B, D) of PEP production by *L. acidophilus* 5e2 as a function of nitrogen source and initial activity of medium. L: linear effect; Q: quadratic effect. First (A, B) and second (C, D) step of CCD.

input variable, *i.e.* PEP synthesis. The conducted analyses showed the linear and square effect of both nitrogen concentration in the culture medium and the initial medium acidity (Fig. 1).

Regression coefficients (Table 4) enabled determining an equation that describes the effect of the values of input variables on PEP synthesis:

$$Y_{PEP} = -782.80 + 13.85X_{2'} - 0.14X_{2'}^2 + 274.54X_{4'} - 24.09X_{4'}^2 - 1.26X_{2'}X_{4'}$$

Table 6
Analysis of variance for the response of the 2^2 for second step of CCD of nitrogen source and initial acidity of medium, $R^2 = 0.8703$, $R^2_{adj} = 0.8239$.

Variables	Sum of square	Degree of freedom	Mean square	*F*-test	*p*-Value
Nitrogen source[a] (1L[b])	3378.73	1	3378.73	28.04	0.000[d]
Nitrogen source[a] (1Q[c])	2693.03	1	2693.03	22.35	0.000[d]
Medium initial acidity (2L[b])	577.84	1	577.84	4.80	0.046[d]
Medium initial acidity (2Q[c])	5304.75	1	5304.75	44.02	0.000[d]
1L[b] by 2L[b]	1529.04	1	1529.04	12.69	0.003[d]
Error	1686.92	14	120.49		
Total	13,001.18	19			

[a] Mixture of vegetable and soymeal peptone in mass ratio 15:7.
[b] L: linear effect.
[c] Q: quadratic effect.
[d] Significant at 95% level.

where Y_{PEP}: quantity of synthesized prolyl endopeptidase (U/mg); $X_{2'}$: concentration of nitrogen compounds in the culture medium (g/l), and $X_{4'}$: initial acidity of the culture medium (pH).

If partial derivatives of the equation describing the impact of experimental parameters on PEP synthesis (Y_{PEP}) equal zero, two subsequent equations may be formulated:

$$13.85 - 0.28X_{2'} - 1.26X_{4'} = 0$$

$$274.54 - 1.26X_{2'} - 48.18X_{4'} = 0$$

These equations enabled calculating optimal values of nitrogen source concentration in the culture medium (g/l) and initial acidity of the culture medium (pH) at $X_{2'} = 26.276$ and $X_{4'} = 5.153$, respectively. For these input values, the determined model predicts the activity of prolyl endopeptidase at 88.89 U/mg, which is higher than the experimentally-determined value of 85.8 U/mg.

For further optimization of the values of the analyzed input parameters in PEP synthesis by *L. acidophilus* 5e2, use was made of the utility function available in the CCD method. The highest and the lowest activity of PEP, accounting for 85.8 and 15.2 U/mg respectively, was ascribed to the maximum (1) and the minimum (0) utility. The function utility, the profile of approximated values of PEP activity and confidence interval were presented in Fig. 2. The best combination of

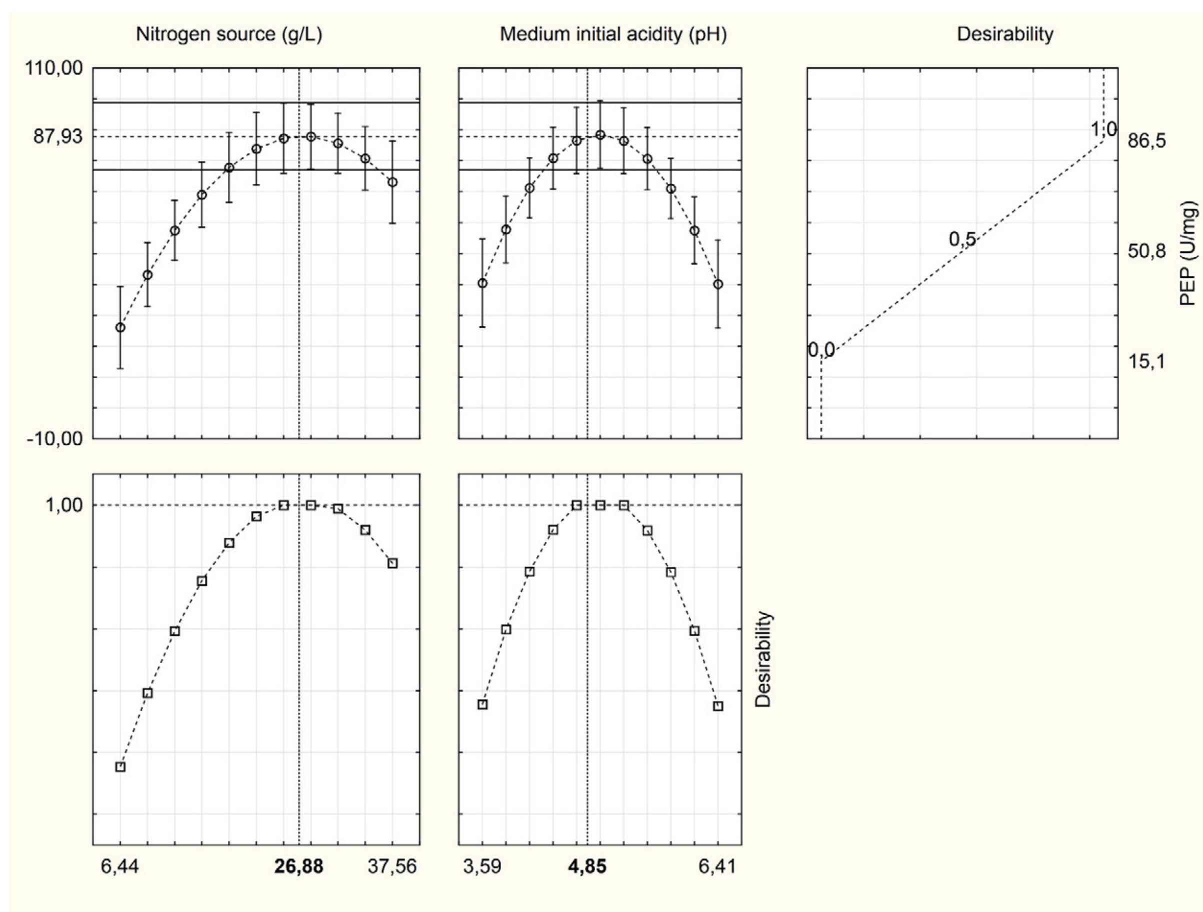

Fig. 2. Profile of predicted values and desirability in the range of nitrogen source concentration (g/l) and initial acidity of medium (pH) studied in the second step of CCD optimization.

the values of input parameters determined using the utility profiles reached 26.88 g/l in the case of nitrogen compounds and pH 4.85 in the case of the initial medium acidity. For these parameters, the activity of synthesized PEP accounted for 88.93 U/mg. The resultant statistical model and regression equation were validated for the values of the variables determined using the utility profiles with values of the remaining variables left at the level of center point values. In the experiment conducted under the optimized conditions, the activity of prolyl endopeptidase synthesized by *L. acidophilus* 5e2 reached 83.54 U/mg, which confirms model correctness.

Results achieved in the study demonstrate that the synthesis of intracellular prolyl endopeptidase is affected by the source of nitrogen and initial acidity of the culture medium. The highest PEP activity was achieved in *L. acidophilus* 5e2 culture incubated onto the medium containing a mixture of vegetable and soymeal peptone as a source of nitrogen. The addition of peptone or a vegetable extract may stimulate the growth of *L. acidophilus* species bacteria, leading to their enhanced proteolytic activity [18]. Previous investigations demonstrated that amino acids and peptides released from proteins of the culture medium by the proteolytic system of lactic fermentation bacteria are indispensable for the growth of microbial cells [19]. The proteolytic system of *L. acidophilus* consists of peptidases and proteases as well as amino acid and peptide transport system including ABC transporters (ATP-binding cassette). Analyses of *L. acidophilus* NCFM genome demonstrate the presence of genes encoding proteases linked with cell membranes (PrtP) and lipoproteins (PrtM) attached to a cell membrane owing to which the high-molecular proteins are degraded extracellularly to peptides and amino acids. Next, they are transported to a cell's interior by permeases and oligopeptide transport system (OPP) [20]. The use of vegetable and soy peptones as sources of protein stimulates cells to synthesize enzymes, including prolyl endopeptidase, that are capable of degrading proline-rich peptides. Also other lactic acid bacteria like *Lactobacillus alimentarius*, *Lactobacillus brevis*, *Lactobacillus sanfranciscensis* or *Lactobacillus hilgardii*, isolated from media containing proline-rich proteins, were shown to display both the activity of prolyl endopeptidase and capability to degrade peptides of plant origin [13,21].

Lactic acid bacteria naturally acidify the environment they colonize by producing such metabolites as lactic acid or acetic acid. Species of these bacteria isolated from dairy products, but also from baker's starters, are able to grow in the medium with acidity of pH 4.0–5.0. Some strains of *L. acidophilus* are characterized by high resistance to environment acidification and may grow even at pH 4.0 [22]. Therefore, culture media pre-acidified to pH 5.0–5.7 are recommended for cultures of lactic acid bacteria [23]. Worthy of notice is, however, that the proline-specific enzymes isolated from *L. sanfranciscensis* CB1 exhibited beneficial activity at pH 5.0–5.5 [24]. Our experiments confirm the capability of *L. acidophilus* 5e2 to synthesize prolyl endopeptidase in the environment with pH 5.0.

4. Conclusions

The reported study demonstrated that the synthesis of prolyl endopeptidase depended mainly on the type and concentration of nitrogen sources available in the culture medium as well as on the initial acidity of the medium. The use of the optimized parameters of *L. acidophilus* 5e2 culture enabled a 3-fold increase in the activity of the synthesized prolyl endopeptidase, compared to the control culture on the MRS-broth.

Financial support

Agency/Institution: The National Science Centre in Poland. Program Financial support: Research Project. Project number: N N312 170739.

Acknowledgments

This study was financed by governmental funds for science in the years 2010–2013 as a Research Project no. N N312 170739.

Author contribution

Proposed the theoretical frame: BB; Conceived and designed the experiments: BB; Contributed reagents/materials/analysis tools: The National Science Centre in Poland; Wrote the paper: BB; Performed the experiments: BB, ML; and Analyzed the data: BB, ML.

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