International Dairy Journal 19 (2009) 236–243

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Optimization of the associative growth of novel yoghurt cultures in the production of biomass, β-galactosidase and lactic acid using response surface methodology

Canan Tari [a,*], Fatma Isık Ustok [b], Sebnem Harsa [a]

[a] Department of Food Engineering, Izmir Institute of Technology, Urla, Izmir, Turkey
[b] Biotechnology and Bioengineering Programme, Izmir Institute of Technology, Izmir, Turkey

ARTICLE INFO

Article history:
Received 24 March 2008
Received in revised form
21 October 2008
Accepted 31 October 2008

ABSTRACT

The associative growth of *Streptococcus thermophilus* 95/2 (St 95/2) and *Lactobacillus delbrueckii* ssp. *bulgaricus* 77 (Lb 77) isolated from the Toros mountain region of Turkey was investigated with respect to lactic acid, biomass and β-galactosidase enzyme production using response surface methodology (RSM). The ratio (St 95/2:Lb 77) of the strains and media formulation had significant effect on all responses ($p < 0.001$). The predicted enzyme activity (2.14 U mL^{-1}), lactic acid (22.50 g L^{-1}) and biomass (7.11 g L^{-1}) production at optimum conditions were very close to the actual experimental values (2.14 U mL^{-1}, 22.94 g L^{-1} and 7.86 g L^{-1}, respectively). The optimum conditions were to use these cultures in a ratio of 1.66:1.62 (St 95/2:Lb 77) in a medium containing whey (5%), corn steep liquor (4%), potassium phosphate (2%) and peptone (2%) at 43 °C for 8 h. The associative growth provided 6.4% and 39% more β-galactosidase activity and 8.73% and 44% more lactic acid compared with the results obtained using pure St 95/2 and Lb 77 strains, respectively.

1. Introduction

Yoghurt, a major food product with an increasing consumption rate due to its health promoting properties, is produced by the action of yoghurt bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) and their metabolites (Kristo et al., 2003). The compounds that provide the typical flavour in yoghurt are lactic acid and volatile organic aroma compounds such as acetaldehyde, diacetyl and acetone (Beshkova et al., 1998). Lactic acid is a valuable product that has several applications in food, chemical and pharmaceutical industries. Its current worldwide production is estimated to be around 120 000 MT y^{-1}, growth being due to its recent potential as major feedstock, derived from renewable carbohydrates by sustainable technologies, to make plastics, fibers, solvents and oxygenated chemicals. This increase has been mainly attributed to the development of new technologies in the fields of separation and purification (Datta and Henry, 2006).

Lactase, i.e., β-galactosidase (E.C. 3.2.1.23), an enzyme produced by lactic acid bacteria (LAB) that hydrolyzes lactose (an abundant disaccharide found in milk) to glucose and galactose, has potential importance in the dairy industry (El Demerdash et al., 2006; Nancip et al., 1999; Vasiljevic and Jelen, 2001, 2002). The nutritional value of lactose is limited due to the fact that a large proportion (50%) of world's inhabitants lacks this enzyme and cannot utilize lactose, thereby developing lactose maldigestion or intolerance (Greenberg and Mahoney, 1982; Somkouti et al., 1996; Vasiljevic and Jelen, 2002). This, however, creates a potential market for the application of β-galactosidase.

Although β-galactosidase has been found in several biological systems, yeast, molds and bacteria are the main sources for commercial purposes (Vasiljevic and Jelen, 2001). In recent years, lactic acid bacteria have gained more importance due to generally regarded as safe (GRAS) status and much research has been conducted on their use (Vasiljevic and Jelen, 2001, 2002, 2003). Among lactic acid bacteria, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are the highest β-galactosidase producers (Greenberg and Mahoney, 1982; Shah and Jelen, 1991; Vasiljevic and Jelen, 2001). The β-galactosidase of these cultures has been characterized, showing greater stability at high temperatures (50 °C) than the β-galactosidase obtained from *Kluyveromyces lactis* (Greenberg and Mahoney, 1982; Kreft and Jelen, 2000). At these conditions (neutral pH and high temperatures), hydrolysis of lactose can be achieved rapidly without growth of undesirable microorganisms (Greenberg and Mahoney, 1982; Vasiljevic and Jelen, 2001). However, LAB cultures are not as high biomass producers as are yeasts, and would not produce lactase at high enough concentrations to be commercially viable as an enzyme producer (Gekas and Lopez-Leiva, 1985).

* Corresponding author. Tel.: +90 232 7506316; fax: +90 232 7506196.
E-mail address: canantari@iyte.edu.tr (C. Tari).

0958-6946/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.idairyj.2008.10.009

In this particular study, a strategy to overcome this problem was to use the advantage of their associative growth properties in the increase of the enzyme yield. The growth between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* is defined as associative since each organism can grow separately and when grown together provides compounds which benefit the other (Tamime and Robinson, 1999). This strategy would not only enhance the product yield, but also provide a diverse range of metabolite production that could be of interest for further research. Most studies described in literature investigated the effect of symbiotic relations on rheological and microbiological properties of dairy products only (Sodini et al., 2005). Furthermore, most of the studies on β-galactosidase production were carried using single strains (Greenberg and Mahoney, 1982; Vasiljevic and Jelen, 2001, 2002, 2003). The originality of this study, however, lies in investigating the impact of a mixed culture on β-galactosidase, lactic acid and biomass production.

With this perspective, 136 LAB cultures isolated from Toros mountain region of Turkey were screened and, from these isolates, *S. thermophilus* 95/2 (coded as St 95/2) and *L. delbrueckii* ssp. *bulgaricus* 77 (coded as Lb 77) were determined as potential lactic acid and β-galactosidase enzyme producers (unpublished data). The aim of this study was the optimization of the fermentation conditions of pure and mixed cultures of Lb 77 and St 95/2 using response surface methodology with respect to biomass formation, lactic acid and β-galactosidase enzyme production. The optimization studies were carried out in shake flasks, and data obtained were adapted to bioreactor scale under controlled environment.

2. Material and methods

2.1. Microorganisms and culture propagation

L. delbrueckii ssp. *bulgaricus* 77 and *S. thermophilus* 95/2 were isolated from traditional yoghurt samples of Toros mountain region of Turkey. Phenotypic and genotypic characterizations of the cultures were performed by the Molecular Food Biotechnology research group at Izmir Institute of Technology (Erkuş et al., 2006). Stock cultures of these strains were prepared by mixing 0.5 mL of activated culture with 0.5 mL of glycerol-broth solution (40%) and stored at −80 °C in sterile cryovials. Culture propagation was carried out by transferring 10 μL of the stock culture of St 95/2 and Lb 77 into 10 mL of sterile M17 and MRS broth, respectively, and incubating overnight at 43 °C.

2.2. Fermentation

Fermentations were carried out both in shake flasks and bioreactor. Optimization studies were performed in 250 mL Erlenmeyer flasks containing 100 mL of sterilized broths of skim milk (8–16%, w/v) for Lb 77 and 100 mL fermentation medium containing whey (0–5%, w/v), corn step liquor (CSL) (Sigma C 4648, Sigma-Aldrich Inc. St Louis, MO, USA) (0–5%, w/v), peptone (2%, w/v) and potassium phosphate (2%, w/v) for St 95/2. Media used during these optimization studies were chosen according to the results of preliminary screening experiments based on medium and culture selection (unpublished work). These media were inoculated with predefined inoculum ranges of propagated culture (2–4%, v/v which were 1.20×10^8–2.40×10^8 cfu mL^{-1} for Lb 77 and 1.40×10^8–2.80×10^8 cfu mL^{-1} for St 95/2) and incubated at predefined temperatures (35–43 °C for Lb 77 and 35–45 °C for St 95/2) for 8 h without agitation as described in the experimental design Section 2.8 (Tables 1 and 2).

During the fermentation, pH in shake flasks could not be controlled due to the risk of contamination and the high number of runs given by the experimental design. In shake flasks, the initial pH of the fermentation medium was 6.5–6.8 for Lb 77 and 7.0–7.6 for St 95/2 (depending on the medium formulation).

The associative relationship of St 95/2 and Lb 77 was determined according to the results obtained from the optimization studies described above. Two types of optimized media formulations were used in this fermentation. Medium A was prepared by reconstituting 8 g skim milk powder (obtained from spray-dried pasteurized milk, Pınar Süt Inc. Izmir, Turkey) in 100 mL deionized water and Medium B was prepared by reconstituting sweet whey powder (5 g; obtained from spray-dried sweet whey: Pınar Süt Inc.), CSL (4 g), potassium phosphate (2 g) and peptone (2 g) in 100 mL deionized water. These media were inoculated with activated cultures (Lb 77 and St 95/2) as described in experimental design (Table 3). The initial pH for Medium A was 6.78 and for Medium B was pH 7.2.

To investigate the effect of pH control on β-galactosidase activity and lactic acid production, bioreactor studies (2.5 L) were performed at optimized conditions with and without pH control using an Infors-Minifors Bioreactor (Infors HT, Bottmingen, Switzerland) (5 L) for pure and mixed cultures.

2.3. Disruption of microbial biomass

Since β-galactosidase from both *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* is an intracellular enzyme, lysozyme enzyme treatment was used for enzyme extraction. For the extraction procedure, 10 mL of fermentation broth was harvested by centrifugation at $2800 \times g$ for 15 min at 4 °C followed by washing the pellet with 10 mL of 0.05 M sodium phosphate buffer (pH 7) and centrifuging at $2800 \times g$ for another 15 min. After the washing step, the pellet was resuspended in 4.5 mL of the same buffer followed by vigorous vortexing. Afterwards, 100 mg of lysozyme (Sigma L 6876, Sigma-Aldrich Inc. St Louis, MO, USA) was added to this solution and incubated at 37 °C for 15 min. After this, 0.5 mL of 4 M NaCl solution was added and incubated further at 37 °C for another 50 min followed by centrifugation at 2800 × g for 15 min. Following centrifugation, the supernatant was used for the enzyme assay.

2.4. Enzyme assay

The activity of β-galactosidase was assayed according to the procedure described in the *Food chemical codex* (National Academy of Sciences, 1996). The chromogenic substrate o-nitrophenol-β-D-galactopyranoside (ONPG, Sigma N 1127, Sigma-Aldrich Inc.) (8.3×10^{-3} M) dissolved in 0.05 M sodium phosphate buffer (pH 7.0) was used. The amount of substrate and enzymes used were 2 mL and 0.5 mL, respectively. At time zero, 0.5 mL of enzyme solution was added to the ONPG solution and incubated for 15 min. The assay was stopped by the addition of 0.5 mL 10% sodium carbonate, and the absorbance was determined at 420 nm. One unit was defined as the quantity of enzyme that would liberate 1 mM of o-nitrophenol from ONPG per minute under the assay conditions. Units were calculated using the following equation:

$$\text{Unit mL}^{-1} = A \times \text{dilution factor} / (\epsilon \times \text{time} \times \text{enzyme solution}) \quad (1)$$

where A was the absorbance at 420 nm, dilution factor was the fold dilution of the enzyme solution, enzyme solution was the amount of enzyme (mL) undergoing the reaction, ϵ was the extinction coefficient (determined from the o-nitrophenol standard curve) and time was the incubation time (15 min).

Table 1
Optimization of Lb77 culture conditions for production of β-galactosidase activity, biomass and lactic acid using a face-centered central composite design matrix.

Run	Temperature (°C)	Inoculum (cfu mL^{-1})	Skim milk (%)	Activity (U mL^{-1})	Biomass (g L^{-1})	Lactic acid (g L^{-1})
1[a]	39	1.80×10^8	12	1.186	8.35	7.69
2	43	1.80×10^8	12	1.258	9.35	10.45
3[a]	39	1.80×10^8	12	1.179	9.15	7.62
4	35	2.40×10^8	16	0.18	3.35	1.98
5	35	2.40×10^8	8	0.239	4.86	6.06
6	35	1.20×10^8	8	0.121	3.15	5.41
7[a]	39	1.80×10^8	12	1.185	8.35	7.65
8[a]	39	1.80×10^8	12	1.191	8.18	7.63
9	39	1.80×10^8	16	0.957	6.95	4.21
10	35	1.20×10^8	16	0.492	4.35	1.54
11	43	1.20×10^8	8	0.814	5.27	9.27
12	43	2.40×10^8	16	0.873	5.8	7.58
13	43	1.20×10^8	16	0.457	4.51	7.99
14	43	2.40×10^8	8	1.341	9.45	14.44
15	39	1.80×10^8	8	0.997	7.85	8.14
16[a]	39	1.80×10^8	12	1.191	9.16	7.58
17[a]	39	1.80×10^8	12	1.183	8.15	7.61
18	35	1.80×10^8	12	0.549	4.75	2.19
19	39	2.40×10^8	12	1.02	7.65	8.42
20	39	1.20×10^8	12	0.987	6.85	5.97

[a] The average and the standard deviation among the repeated experiments performed at all center points were 1.186 ± 0.005 U mL^{-1}, 8.56 ± 0.47 g L^{-1}, and 7.63 ± 0.037 g L^{-1} for activity (β-galactosidase activity), biomass and lactic acid, respectively.

2.5. Biomass determination

Due to whey or milk protein precipitation at low pH values, biomass data could not be determined adequately. To minimize the contribution of protein precipitates to the biomass weight, sterile media (sterilized at 113 °C for 10 min) used during the fermentation were prepared and their pH were adjusted to the final pH obtained at the end of fermentation. The amount of protein precipitates was determined by centrifugation (at $2800 \times g$ for 15 min) and drying the pellet at 43 °C until constant weight was reached. The biomass, including the precipitate at the end of each fermentation, was centrifuged and dried to constant weight. The biomass (g L^{-1}) was determined by subtracting the amount of protein precipitates (determined from sterile medium) from dried pellet (biomass including the precipitate) obtained at the end of fermentation. This approach provided an approximation of the biomass produced.

2.6. Lactose and lactic acid determination

Lactose and lactic acid determination were performed by using Perkin Elmer HPLC system (Perkin Elmer, Boston, MA, USA) equipped with a pump (PE Series 200), refractive index detector (PE

Table 2
Optimization of St 95/2 culture conditions for production of β-galactosidase activity, biomass and lactic acid using a face-centered central composite design matrix.

Run	Temperature °C	Inoculum (cfu mL^{-1})	Whey (%)	CSL[b] (%)	Activity (U mL^{-1})	Biomass (g L^{-1})	Lactic acid (g L^{-1})
1[a]	40	2.10×10^8	2.5	2.5	1.796	6.75	13.45
2[a]	40	2.10×10^8	2.5	2.5	1.805	6.80	13.40
3	35	2.10×10^8	2.5	2.5	0.485	4.15	9.90
4	45	1.40×10^8	5	5	0.755	8.50	19.92
5[a]	40	2.10×10^8	2.5	2.5	1.792	6.76	13.28
6	45	1.40×10^8	5	0	0.014	3.55	1.49
7	35	2.80×10^8	0	0	0.091	1.75	0
8[a]	40	2.10×10^8	2.5	2.5	1.764	6.87	13.36
9	40	2.10×10^8	5	2.5	0.964	7.75	16.69
10	40	2.10×10^8	2.5	5	0.336	7.35	13.46
11	35	1.40×10^8	5	0	0.011	2.95	1.05
12	35	2.80×10^8	5	0	0.061	3.85	2.54
13	45	2.80×10^8	5	5	1.196	9.65	22.75
14	40	2.10×10^8	0	2.5	0.196	2.40	9.56
15[a]	40	2.10×10^8	2.5	2.5	1.794	6.85	13.48
16	45	1.40×10^8	0	0	0.056	1.70	0
17	35	2.80×10^8	5	5	0.628	8.17	10.62
18	45	2.80×10^8	0	0	0.062	1.40	0
19	45	2.10×10^8	2.5	2.5	1.133	5.65	11.75
20[a]	40	2.10×10^8	2.5	2.5	1.828	6.78	13.45
21	45	1.40×10^8	0	5	0.17	3.80	11.78
22	40	1.40×10^8	2.5	2.5	1.160	4.35	7.86
23	35	1.40×10^8	5	5	0.316	5.50	8.38
24	35	1.40×10^8	0	5	0.097	2.85	7.81
25	35	1.40×10^8	0	0	0.062	1.05	0
26	45	2.80×10^8	0	5	0.473	5.35	12.14
27	40	2.80×10^8	2.5	2.5	2.027	7.95	14.28
28	40	2.10×10^8	2.5	0	0.029	4.95	2.85
29	35	2.80×10^8	0	5	0.206	3.20	10.54
30	45	2.80×10^8	5	0	0.022	4.72	3.71

[a] The average and the standard deviation among the repeated experiments performed at all center points were 1.796 ± 0.021 U mL^{-1}, 6.80 ± 0.048 g L^{-1}, and 13.41 ± 0.076 g L^{-1} for activity (β-galactosidase activity), biomass and lactic acid, respectively.
[b] CSL: corn steep liquor.

Table 3
Optimization of medium type and inoculum amount of St 95/2 and Lb 77 for production of β-galactosidase activity, biomass and lactic acid using a face-centered central composite design matrix.

Run	St 95/2 (cfu mL^{-1})	Lb 77 (cfu mL^{-1})	Medium	Activity (U mL^{-1})	Biomass (g L^{-1})	Lactic acid (g L^{-1})
1[a]	1.24×10^8	1.08×10^8	A	0.748	8.56	8.18
2[b]	1.24×10^8	1.08×10^8	B	1.748	6.75	21.33
3[b]	1.24×10^8	1.08×10^8	B	1.723	6.87	21.35
4[a]	1.24×10^8	1.08×10^8	A	0.754	8.78	8.16
5[a]	1.24×10^8	1.08×10^8	A	0.758	9.02	8.17
6	1.86×10^8	1.08×10^8	A	0.806	10.12	9.85
7[a]	1.24×10^8	1.08×10^8	A	0.752	8.93	8.18
8	1.86×10^8	1.62×10^8	B	2.058	7.18	18.43
9	0.62×10^8	1.08×10^8	A	0.538	6.92	5.88
10	1.24×10^8	1.62×10^8	B	2.144	6.45	24.86
11[a]	1.24×10^8	1.08×10^8	A	0.754	8.97	8.15
12	0.62×10^8	1.62×10^8	A	0.693	7.75	6.02
13	0.62×10^8	0.54×10^8	A	0.655	6.90	7.07
14	0.62×10^8	1.62×10^8	B	1.531	4.65	20.45
15	0.62×10^8	1.08×10^8	B	1.235	4.21	18.64
16	1.86×10^8	1.62×10^8	A	1.013	10.25	11.31
17	1.86×10^8	1.08×10^8	B	1.739	5.97	22.31
18	1.24×10^8	0.54×10^8	B	1.327	5.10	19.16
19[b]	1.24×10^8	1.08×10^8	B	1.740	6.23	21.30
20	1.24×10^8	1.62×10^8	A	0.734	10.18	8.75
21	1.86×10^8	0.54×10^8	A	0.544	7.15	5.17
22	1.24×10^8	0.54×10^8	A	0.495	6.85	5.29
23[b]	1.24×10^8	1.08×10^8	B	1.734	6.23	21.39
24	0.62×10^8	0.54×10^8	B	1.124	4.87	14.76
25	1.86×10^8	0.54×10^8	B	1.256	5.26	16.70
26[b]	1.24×10^8	1.08×10^8	B	1.637	6.20	21.33

[a] The average and the standard deviation among the repeated experiments performed at all center points using Medium A were 0.753 ± 0.004 U mL^{-1}, 8.85 ± 0.186 g L^{-1}, and 8.61 ± 0.013 g L^{-1} for activity (β-galactosidase activity), biomass and lactic acid, respectively.
[b] The average and the standard deviation among the repeated experiments performed at all center points using Medium B were 1.7164 ± 0.045 U mL^{-1}, 6.45 ± 0.326 g L^{-1}, and 21.34 ± 0.033 g L^{-1} for activity (β-galactosidase activity), biomass and lactic acid, respectively.

Series 200) and interface (PE Series 900). Samples (20 μL) were injected through the injection port. The separation was performed with an Aminex HPX-87H ion exclusion column (300 × 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA). The column was heated at 45 °C in column oven (Metatherm, Lake Forest, CA, USA). The conditions applied were: 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1}. Data acquisition and peak processing were performed with Total Chrom Workstation ver. 6.2.1 software (Perkin Elmer). Peak identification was based on retention times and peak quantification was based on the external standard method. In this method, the reference standards were chosen to be the same as the solutes in the sample and given to the system separately. Initial and residual lactose amounts were recorded and used in calculations of yield coefficients.

2.7. Microbiological analysis

To investigate the effect of associative growth on final cell counts, microorganisms were enumerated using differential media (Hekmat and McMahon, 1997; Shah, 2000; Van de Casteele et al., 2006; Yüceer et al., 2001). *L. delbrueckii* ssp. *bulgaricus* was enumerated on MRS agar (pH 5.4) after anaerobic incubation at 43 °C for 48 h (at pH 5.4 no growth of *S. thermophilus* was observed). *S. thermophilus* was counted on M17 agar after incubation at 43 °C for 48 h.

2.8. Experimental design and statistical analysis

Three different optimization studies were performed using the statistical software called Design Expert 7.0 trial version (Stat-Ease Inc. Minneapolis, MN, USA). In the first optimization, effects of independent variables including temperature (X_1), inoculum (X_2) and skim milk concentration (X_3) were investigated on the responses of lactic acid, biomass and β-galactosidase activity using Lb 77. A face-centered composite design (CCD) with three factors was used (Table 1). In the second optimization, effects of independent variables temperature (X_1), inoculum (X_2), whey concentration (X_3) and corn steep liquor (CSL) (X_4) were investigated on the responses of lactic acid, biomass and β-galactosidase activity using St 95/2. A face-CCD with four factors was used (Table 2).

Based on the results obtained from the optimization studies described above, a third optimization study was performed to investigate the associative relationship between Lb 77 and St 95/2. Effects of independent variables such as inoculum ratio of St 95/2 (X_1), inoculum ratio of Lb 77 (X_2) and media formulation (X_3) on the responses of lactic acid, biomass and β-galactosidase activity were investigated (Table 3).

In all optimization studies, analysis of data and generation of response surface plots were achieved with the statistical software Design Expert (trial version). The response variables in three sets of experiments were lactic acid, biomass and β-galactosidase activity. A second-order polynomial equation including the interactions was fitted to the response data by multiple regression procedure, after running the experiments and measuring the response variables:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j + \epsilon \quad (2)$$

where Y is the predicted response, k is the number of factor variables, β_0 is the model constant, β_i is linear coefficient, X_i and X_j are independent variables in its coded form, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient and ϵ is the error factor. To code the actual experimental values of the factors in a range of $[-1\ +1]$, the following equation was used:

$$x = [\text{actual} - (\text{low level} + \text{high level})/2]/(\text{high level} - \text{low level})/2 \quad (3)$$

The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and

Table 4
R^2, Adjusted R^2 and Predicted R^2 values of optimization studies.

Parameter	Activity			Lactic Acid			Biomass		
	1st optimization	2nd optimization	3rd optimization	1st optimization	2nd optimization	3rd optimization	1st optimization	2nd optimization	3rd optimization
R^2	0.989	0.95	0.977	0.901	0.975	0.96	0.957	0.95	0.954
Adjusted R^2	0.98	0.91	0.966	0.882	0.965	0.942	0.919	0.891	0.932
Predicted R^2	0.851	0.784	0.916	0.808	0.923	0.807	0.759	0.765	0.861

interaction terms were determined. The significances of all terms in the polynomial were judged statistically according to their p-values.

3. Results and discussion

3.1. Optimization using pure cultures

Optimization studies using pure strains of Lb 77 and St 95/2 were performed as described in Section 2.8. The experimental runs with response variables (β-galactosidase activity, lactic acid and biomass) are presented in Tables 1 and 2. The results of ANOVA for all three responses show that the individual models were highly significant for each experimental design (data not shown). The R-square values are given in Table 4 for β-galactosidase activity, lactic acid and biomass, respectively, indicating a good agreement between experimental and predicted values. The "predicted R^2" was in agreements with "Adjusted R^2".

According to the CCD (Table 1) and response surface plots (not shown) for Lb 77, maximum β-galactosidase activity, biomass and lactic acid could be attained at higher temperatures and inoculum amounts at a skim milk concentration of 8–8.5%. Therefore, the optimum conditions predicted by the software for maximum β-galactosidase activity (1.36 U mL^{-1}), biomass (9.43 g L^{-1}) and lactic acid (13.06 g L^{-1}) were to use a temperature of 43 °C, at an inoculum of 2.40×10^8 cfu mL^{-1} and skim milk concentration of 8.0% (Table 5). The final pH of the fermentations ranged between pH 3.95 and 4.53, depending on the growth of Lb 77.

From the CCD carried out using St 95/2, maximum β-galactosidase activity could be attained at higher temperatures and inoculum amounts with whey and corn steep liquor (CSL) concentrations of 2.5% (Table 2). However, maximum lactic acid and biomass production could be obtained at higher temperatures and inoculum amounts with maximum whey and CSL concentrations of 5%. Based on these, optimum conditions for maximum β-galactosidase activity (2.03 U mL^{-1}), biomass (9.07 g L^{-1}) and lactic acid (22.76 g L^{-1}) production were defined as a temperature of 44 °C with an inoculum concentration of 3.87% at whey and CSL concentrations of 5% and 4.16%, respectively (Table 5).

Comparing the results of these optimization studies show that St 95/2 produces 1.49 times more β-galactosidase and 1.74 times more lactic acid than Lb 77 at predicted optimum conditions. The final pH of the fermentations ranged between pH 4.79 and 7.36, depending on the growth of St 95/2.

3.2. Optimization using mixed cultures

Based on the results obtained from the optimization studies described above, a third optimization study was performed to investigate the effect of the associative growth of Lb 77 and St 95/2 on the response parameters as a strategy to increase the product yields. The effects of inoculum amounts of St 95/2 and Lb 77 and media formulations were investigated on the response variables (lactic acid, biomass and β-galactosidase activity) using a CCD. Two types of media formulations Medium A (8% skim milk) and Medium B (5% whey, 4% corn steep liquor, 2% potassium phosphate and 2% peptone), obtained from the previous optimization studies were used. It is important to note that the composition of corn steep liquor can vary depending on the source and the process; however, in this study it was obtained from a reliable source with a certain standard (Section 2.2). Therefore, this issue was not of a concern, but could be one in large-scale productions requiring special attention.

The experimental runs with response variables are presented in Table 3. The results of ANOVA for all three responses revealed that the individual models were highly significant ($p < 0.0001$, data not

Table 5
Experimental and predicted values[a] for β-galactosidase activity, lactic acid and biomass at optimum conditions.

(a) Validation experiments for first optimization					
Running order	Inoculum (cfu mL^{-1})	Skim milk (%, w/v)	Activity (U mL^{-1})	Lactic acid (g L^{-1})	Biomass (g L^{-1})
1	2.40×10^8	8	1.303 (1.363)	12.99 (13.06)	8.96 (9.43)
2	2.34×10^8	8	1.269 (1.360)	12.05 (12.94)	9.65 (9.44)
3	2.39×10^8	8.15	1.294 (1.322)	12.87 (12.96)	9.75 (9.40)
4	2.40×10^8	8.63	1.332 (1.370)	13.07 (12.87)	10.12 (9.45)
(b) Validation experiments for second optimization					
Running order	Inoculum (cfu mL^{-1})	CSL (%, w/v)	Activity (U mL^{-1})	Lactic acid (g L^{-1})	Biomass (g L^{-1})
1	2.71×10^8	4.16	1.986 (2.027)	20.85 (22.76)	9.76 (9.08)
2	2.69×10^8	4.07	2.015 (2.032)	21.55 (22.77)	10.15 (9.06)
3	2.76×10^8	4.34	1.992 (2.043)	19.98 (22.76)	10.28 (9.04)
4	2.73×10^8	4.17	2.032 (2.045)	21.37 (22.76)	8.94 (9.11)
(c) Validation experiments for third optimization					
Running order	St 95/2 (cfu mL^{-1})	Lb 77 (cfu mL^{-1})	Activity (U mL^{-1})	Lactic acid (g L^{-1})	Biomass (g L^{-1})
1	1.66×10^8	1.62×10^8	2.162 (2.144)	23.85 (22.52)	7.38 (7.09)
2	1.61×10^8	1.62×10^8	2.108 (2.134)	22.61 (22.50)	8.05 (7.14)
3	1.61×10^8	1.62×10^8	2.126 (2.135)	22.18 (22.47)	7.89 (7.09)
4	1.66×10^8	1.62×10^8	2.153 (2.144)	23.12 (22.53)	8.12 (7.12)

[a] Predicted values are shown in parenthesis. Activity: β-galactosidase activity, CSL: corn steep liquor. Temperature conditions were 43, 44 and 43 °C for first, second and third optimization, respectively. Whey (5%) and Medium B were used in validation experiments of second and third optimization, respectively.

shown). The *R*-square values are presented in Table 4. The model equations for β-galactosidase enzyme ($Y_{\beta\text{-gal}}$), lactic acid (Y_L) and ln (biomass) (Y_b) with the coefficients in coded units of factors are given below:

$$Y_{ß\text{-gal}} = 0.88 + 0.036X_1 + 0.27X_2 + 0.37X_3$$

$$Y_L = 14.17 + 0.39X_1 + 3.16X_2 + 5.21X_3$$

$$Y_b = 7.68 + 1.03X_1 + 0.71X_2 - 1.23X_3 + 0.77X_1X_2 - 0.25X_1X_3 - 0.20X_2X_3 - 0.77X_1^2 - 0.43X_2^2$$

From the results, maximum β-galactosidase activity and lactic acid formation was attained in Medium B with an inoculation ratio of 1.24:1.62 (St 95/2:Lb 77). Maximum biomass, however, was obtained in Medium A, with an inoculation ratio of 1.86:1.62 (St 95/2:Lb 77). Finally, the optimum conditions determined suggested use of these two cultures together in a ratio of 1.66:1.62 (St 95/2:Lb 77) in Medium B for maximum β-galactosidase (2.16 U mL^{-1}) and lactic acid (22.52 g L^{-1}) production. Under these circumstances, maximum biomass achieved was around 7.11 g L^{-1}. Since the optimum condition for maximum biomass production in Medium A resulted in lower enzyme activity and lactic acid formation, this condition was not considered in the validation experiments. The final pH of the fermentation medium ranged for Medium A and Medium B between, 4.27–4.57 and 4.92–5.21, respectively.

To validate the adequacy of the models, a total of three verification experiments that were repeated four times were carried out at the predicted optimum conditions for each optimization (Table 5). As seen from the results, there was a good correlation between the experimental and predicted values. Overall, the associative growth of these cultures provided 6.4% and 39% more β-galactosidase activity and 8.73% and 44% more lactic acid compared with the results obtained using pure St 95/2 and Lb 77 strains, respectively (these numbers are based on the average actual validation experiments conducted under the defined optimum conditions, Table 5). Furthermore, this relationship provided 20.67% and 19.31% less biomass, 19.46% and 35.71% more lactic acid and 17.13% and 29.46% more enzyme yield compared with the use of pure St 95/2 and Lb 77 strains, respectively (Table 6). Considering the productivities (Table 6), associative growth provided 8.7% and 44.44% more lactic acid, 6.4% and 39.32% more β-galactosidase enzyme than pure St 95/2 and Lb 77 strains, respectively. As it is obvious from the given numbers, the effect of the associative growth was more pronounced on Lb 77 than St 95/2. At large scale, these percentages can be considered as significant enhancement. In fact, this has been proven by a two-sample *t*-test by Minitab 14 statistical software (Minitab Inc., PA, USA), which confirmed the significant improvements (data not shown).

To assess the contribution of each strain, differential media were used to enumerate their numbers in the bulk mixture. In other words, the fractions of viable cell counts of individual strains in the bulk mixture were determined. From the cell count fractions, 70–80% of strain St 95/2 and 20–30% of strain Lb 77 contributed to biomass, lactic acid and β-galactosidase activity (data not shown). This was also partly confirmed by the individual and associative specific growth rates of the strains under the optimum conditions. In fact, the specific growth rates of pure Lb 77 and St 95/2 under optimized conditions (first and second optimization) were 0.44 and 0.717 h^{-1}, respectively. However, the relationship under the third optimization condition increased the specific growth rates for both strains by 28% to 0.62 h^{-1} and 1.00 h^{-1}, respectively.

The increases in the parameters discussed above could be mainly attributed to the associative growth characteristics observed among these cultures. It has been reported in a number of studies that the interaction between these two organisms could be attributed to the mechanism of stimulation of the *Streptococcus* through the peptides released by the lactobacilli (Amoroso and Manca de Nadra, 1990; Rajagopal and Sandine, 1990; Tamime and Robinson, 1999). It is also recognized that *L. delbrueckii* ssp. *bulgaricus* could produce more proteolytic enzymes than *S. thermophilus*. Hence, *S. thermophilus* might benefit from the stronger activity of the *L. delbrueckii* ssp. *bulgaricus* and in return produce certain compounds (such as formate) that can stimulate the growth of *L. delbrueckii* ssp. *bulgaricus* (Tamime and Robinson, 1999). All these activities may take place during the incubation period and can be reflected in the amounts of the products such as β-galactosidase, lactic acid and biomass. Although, in industrial enzyme and lactic acid productions, this could be an important strategy in increasing yields of these products, there is still a lot more work needed to unravel what exactly the promoting effects are.

3.3. Effect of pH on β-galactosidase activity and lactic acid production

To investigate the effect of pH control on β-galactosidase activity and lactic acid production, bioreactor studies were performed with and without pH control for Lb 77, St 95/2 and Lb 77 plus St 95/2 according to the results obtained from optimization studies. In the first experiments, pH adjustment was not considered whereas, in the second experiments, pH was adjusted to 6.2 ± 0.1 by automatic addition of 2 M NaOH. These experiments were repeated twice. Fermentation was carried out for 8 h and samples were taken every 2 h for the analysis of enzyme activity and lactic acid. The duration of 8 h was determined in the initial shake flask experiments to result in maximum β-galactosidase activity. Since the primary goal of this study was the optimization of β-galactosidase activity rather than lactic acid production, this time point was taken as the actual fermentation time.

As it is seen from Fig. 1a, β-galactosidase activity was reduced 1.58 times for Lb 77 strain at the constant pH, 6.2, at the end of fermentation. Lactic acid results were also lower when compared with the results obtained at uncontrolled conditions (Fig. 1b). However, for St 95/2, β-galactosidase activity was increased by 3.37% when pH was controlled at 6.2, whereas lactic acid level was reduced at this condition. When both strains were used in combination, enzyme activity and lactic acid were reduced by 3.92% and 26%, respectively at a constant pH of 6.2. These observations could be easily linked to the natural growth requirements of these

Table 6
Yield coefficients and productivities for optimization studies at optimum conditions[a] performed for pure strains (Lb 77 and St 95/2 separately) and mixed strains (Lb 77 and St 95/2).

Optimization	Yield			Productivity	
	Yx/s	*Yp*1/s	*Yp*2/s	Activity (U mL^{-1} h^{-1})	Lactic acid (g L^{-1} h^{-1})
First optimization	0.347 ± 0.017	0.459 ± 0.028	46.97 ± 3.18	0.162 ± 0.003	1.593 ± 0.058
Second optimization	0.353 ± 0.022	0.575 ± 0.035	55.18 ± 2.08	0.250 ± 0.003	2.617 ± 0.088
Third optimization	0.28 ± 0.012	0.714 ± 0.019	66.59 ± 0.59	0.267 ± 0.003	2.867 ± 0.089

[a] *Yx*/s = biomass yield coefficient (g biomass g^{-1} lactose); *Yp*1/s = lactic acid yield coefficient (g lactic acid g^{-1} lactose); *Yp*2/s = activity (β-galactosidase) yield coefficient (U activity g^{-1} lactose); Values are based on the mean ± SD of four validation experiments shown in Table 5.

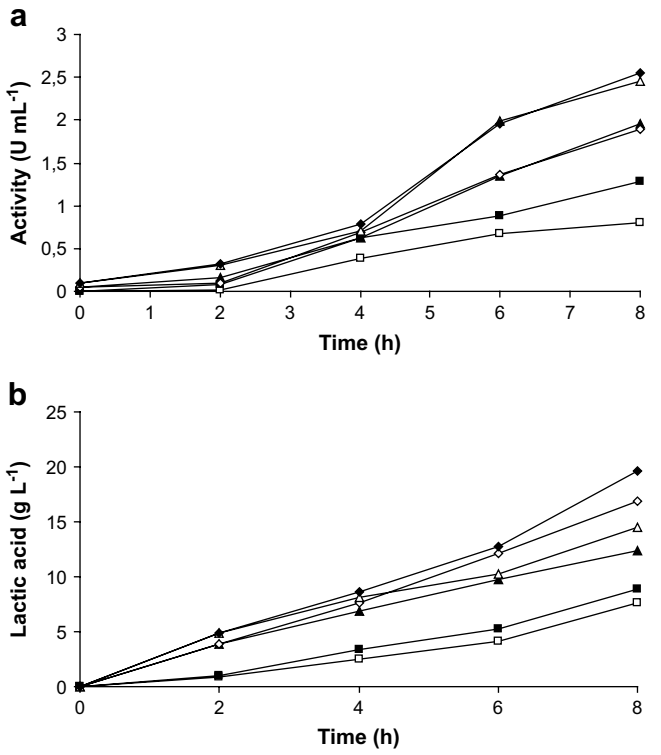

Fig. 1. Enzyme activity (β-galactosidase activity) (a) and lactic acid production (b) under controlled pH (pH 6.2; □, ▲, △) and uncontrolled pH (■, ◇, ◆) for Lb 77 (□, ■), St 95/2 (▲, ◇) and Lb 77 and St 95/2 (△, ◆) in a bioreactor. Mean standard deviations for activity and lactic acid were 0.073 and 1.52, respectively.

individual strains. It has been reported that *S. thermophilus* grows better close to neutral pH (pH 6.8) and is more acid-sensitive, compared with *L. delbrueckii* ssp. *bulgaricus*, which is more acid-tolerant, growing even below pH 5.6 (Ademberg et al., 2003; Hekmat and McMahon, 1997; Kreft et al., 2001; Tamime, 2006; Vasiljevic and Jelen, 2001). Therefore, at a pH of 6.2, *S. thermophilus* grows more rapidly than *L. delbrueckii* ssp. *bulgaricus*. In fact, this is reflected in the data given above. When used in combination, however, the adverse effect of pH (pH 6.2) on *L. delbrueckii* ssp. *bulgaricus* is partly compensated by the positive effect on *S. thermophilus* with respect to enzyme production. Another issue is that the metabolic pathways for lactic acid or production of other metabolites are strongly controlled by pH. This is emphasized for pure as well as for mixed strains. Therefore, in the light of above discussions, it is recommended to apply an uncontrolled pH environment by letting the natural fermentation take over its own course.

This study clearly indicated that it is more efficient to use both strains in combination as opposed to the traditional single organism system often practiced in industrial production of lactic acid and β-galactosidase enzyme. Comparison of the current optimization results with various literature results obtained using pure strains indicated that the present results were considerably higher with respect to β-galactosidase activity (Gaudreau et al., 2005; Geciova et al., 2002; Gueimonde et al., 2002; Montanari et al., 2000). These findings were also in good correlation with a study conducted by Altıok et al. (2006) and Büyükkileci and Harsa (2004) who investigated the lactic acid formation characteristics of *Lactobacillus casei* NRRL B-441. Since there is no study (that we are aware of) conducted on the associative growth relation of the mixed culture with respect to β-galactosidase production, a direct comparison with the literature with this respect could not be made.

4. Conclusion

In this study, the optimum conditions for the production of biomass, lactic acid and β-galactosidase of new strains (St 95/2 and Lb 77) in pure as well as mixed forms were determined using response surface methodology. The associative relationship of these two strains provided higher product yields.

Acknowledgements

Financial support of Turkish Scientific and Technical Research Council (TUBİTAK-TOVAG 1040270) and State Plan Organization (DPT-2005 K 120570) are gratefully acknowledged.

References

Ademberg, K., Kask, S., Laht, T.M., Paalme, T., 2003. The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. International Journal of Food Microbiology 85, 171–183.
Altıok, D., Tokatlı, F., Harsa, S., 2006. Kinetic modeling of lactic acid production from whey by *Lactobacillus casei* (NRRL B-441). Journal of Chemical Technology and Biotechnology 81, 1190–1197.
Amoroso, M.J., Manca de Nadra, M.C., 1990. A new mixed culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* isolated from commercial yogurt. Microbiologie, Aliments, Nutrition 8, 105–113.
Beshkova, D., Simova, G., Frengova, G., Simov, Z., 1998. Production of flavour compounds by yoghurt starters. Journal of Industrial Microbiology and Biotechnology 20, 180–186.
Büyükkileci, A.O., Harsa, S., 2004. Batch production of L(+) lactic acid from whey by *Lactobacillus casei* (NRRL B-441). Journal of Chemical Technology and Biotechnology 79, 1036–1040.
Datta, R., Henry, M., 2006. Lactic acid: recent advances in products processes and technologies – a review. Journal of Chemical Technology and Biotechnology 81, 1119–1129.
El Demerdash, H.A., Oxman, J., Heller, K.J., Geis, A., 2006. Yoghurt fermentation at elevated temperatures by strains of *Streptococcus thermophilus* expressing a small heat-shock protein: application of a two-plasmid system for constructing food grade strains of *Streptococcus thermophilus*. Biotechnology Journal 1, 398–404.
Erkuş O., Celik E. S., Yavuzdurmaz, H., Okuklu, B., Harsa, S. Isolation and molecular characterization of artisanal yoghurt starter bacteria, The 20th international ICFMH symposium on food safety and food biotechnology: "Diversity and Global Impact", 2006, Bologna, Italy.
Gaudreau, H., Champagne, C.P., Jelen, P., 2005. The use of cellular extracts of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 to stimulate growth of a probiotic *Lactobacillus rhamnosus* culture in milk. Enzyme and Microbial Technology 36, 83–90.
Geciova, J., Giesova, M., Jelen, P., Plockova, M., 2002. Disruption of *Streptococcus thermophilus* 143 culture by three mechanical methods for increased β-galactosidase activity. Milchwissenschaft 57, 509–511.
Gekas, V., Lopez-Leiva, M., 1985. Hydrolysis of lactose. Process Biochemistry 2, 2–12.
Greenberg, N.A., Mahoney, R.R., 1982. Production and characterization of β-galactosidase from *Streptococcus thermophilus*. Journal of Food Science 47, 1824–1828.
Gueimonde, M., Corzo, N., Vinderola, G., Reinheimer, J., De Los Reyes-Gavilan, C.G., 2002. Evolution of carbohydrate fraction in carbonated fermented milks as affected by β-galactosidase activity of starter strains. Journal of Dairy Research 69, 125–137.
Hekmat, S., McMahon, D., 1997. Manufacture and quality of iron-fortified yogurt. Journal of Dairy Science 80, 3114–3122.
Kreft, M.E., Champagne, C.P., Jelen, P., 2001. Growth of *Lactobacillus* dairy cultures on two different brands of MRS medium. Milchwissenschaft 56, 315–317.
Kreft, M.E., Jelen, P., 2000. Stability and activity of β-galactosidase in sonicated cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 affected by temperature and ionic environment. Journal of Food Science 65, 1364–1368.
Kristo, E., Biliaderis, C.G., Tzanetakis, N., 2003. Modelling of the reological, microbiological and acidification properties of a fermented milk product containing a probiotic strain of *Lactobacillus paracasei*. International Dairy Journal 13, 517–528.
Montanari, G., Zambonelli, C., Grazia, L., Benevelli, M., Chiavari, C., 2000. Release of β-galactosidase from lactobacilli. Food Technology and Biotechnology 38, 129–133.
Nancip, N., Ghoul, M., Larous, L., Nancib, A., Adimi, L., Remmal, M., et al., 1999. Use of date products in production of the thermophilic dairy starter strain *Streptococcus thermophilus*. Bioresource Technology 67, 291–295.
National Academy of Sciences, 1996. Food Chemicals Codex, fourth ed. National Academy Press, Washington, DC, USA, pp. 802–803.
Rajagopal, S.N., Sandine, W.E., 1990. Associative growth and proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in skim milk. Journal of Dairy Science 73, 894–899.
Shah, N.P., 2000. Symposium: probiotic bacteria: selective enumeration and survival in dairy foods. Journal of Dairy Science 83, 894–907.

Shah, N.P., Jelen, P., 1991. Lactase activity and properties of sonicated dairy cultures. Milchwissenschaft 46, 570–573.
Sodini, I., Lucas, A., Tissier, J.P., Corrieu, G., 2005. Physical properties and microstructure of yoghurts supplemented with milk protein hydrolysates. International Dairy Journal 15, 29–35.
Somkouti, G.A., Dominiecki, M.E., Steinberg, D.H., 1996. Sensitivity of *Streptococcus thermophilus* to chemical permeabilization. Current Microbiology 32, 101–105.
Tamime, A.Y., 2006. Manufacture of yogurt. Fermented milks. Society of dairy technology. Blackwell Publishing, London, UK, pp. 64–65.
Tamime, A.Y., Robinson, R.K., 1999. Microbiology of Yoghurt and "Bio" Starter Cultures. Yoghurt Science and Technology, second ed. Woodhead Publishing Ltd./CRC Press, Cambridge, England/Boca Raton, FL, USA, p. 399.
Van de Casteele, S., Vanheuverzwijn, T., Ruyssen, T., Van Assche, P., Swings, J., Huys, G., 2006. Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yoghurt or cheese starters. International Dairy Journal 16, 1470–1476.
Vasiljevic, T., Jelen, P., 2001. Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria. Innovative Food Science and Emerging Technologies 2, 75–85.
Vasiljevic, T., Jelen, P., 2002. Lactose hydrolysis in milk as affected by neutralizers used for the preparation of crude β-galactosidase extracts from *Lactobacillus bulgaricus* 11842. Innovative Food Science and Emerging Technologies 3, 175–184.
Vasiljevic, T., Jelen, P., 2003. Drying and storage of crude β-galactosidase extracts from *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842. Innovative Food Science and Emerging Technologies 4, 319–329.
Yüceer, K.Y., Wilson, J.C., White, H., 2001. Formulations and processing of yogurt affect the microbial quality of carbonated yogurt. Journal of Dairy Science 84, 543–550.