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# Kinetics of β-galactosidase Production by *Lactobacillus bulgaricus* During pH Controlled Batch Fermentation in Three Commercial Bulk Starter Media

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**Abstract**

The potential of bulk starter fermentation strategy for production of a cost-effective and safe source of β-galactosidase was investigated. Three different bulk starter media with different compositions were selected, and an industrial yogurt starter culture strain, *L. bulgaricus* DSM 20081 was cultivated in these media under pH-controlled conditions (pH=5.6) at 43°C. The media consisted of 1) bulk starter medium based on skim milk and whey, 2) bulk starter medium based on whey, and 3) reconstituted skim milk. The kinetic parameters of growth and β-lactic acid production were estimated using the experimental data with the Garcia and Luedeking-Piret models, respectively. β-galactosidase production kinetics was also simulated using appropriate models based on biomass and lactic acid production. Growth in the bulk starter medium based on skim milk and whey resulted in a higher rate of lactic acid production (7.35 ± 0.23 mg lactic acid $ml^{-1}$ media $h^{-1}$) and β-galactosidase activity (800.1± 0.7 nmol ONP $ml^{-1}$ media) compared to other two media ($P$<0.01). Simulation of β-galactosidase production based on the rate of lactic acid production resulted in a very good agreement with the experimental data of all three tested media. The results revealed the potential of bulk starter fermentation strategy and skim milk + whey based medium for in-house and relatively low cost production of food-grade β-galactosidase by dairy plants.

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## 1. Introduction

Developing robust technologies for commercial production of a cost effective source of β-galactosidase (lactase, β-D-galactosidase galactohydrolase, EC 3.2.1.23) is highly desirable because of its wide applications in food and dairy industries [1]. β-galactosidase hydrolyzes lactose into two moieties (glucose and galactose) as a forward reaction and synthesizes galacto-oligosaccharide (trans-galacosylation) as a reverse reaction. Nutritional and technological benefits of prebiotic galactoolighosaccharides and lactose hydrolysis in milk, whey and other dairy products have been clearly demonstrated [2-7]. During the last decade, production of thermostable β-galactosidase from thermophilic microorganisms has gained increasing attention because of superior economical and technological benefits [8-11]. However, explored thermostable β-galactosidases are mainly produced from non-GRAS (generally recognized as safe) microbial sources, and need to be purified for industrial application [12-15]. Therefore, industrial and comercial applications of these thermostable enzymes are highly limited due to high cost of enzyme extraction

and purification.

Lactic acid bacteria have been the focus of extensive research because of their capability for production of thermo-stable [16,17]. The advantage of β-galactosidase from thermophilic lactic acid bacteria, e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, is its GRAS status as well as high activity and stability at temperatures about 55-60°C [18]. However, few works have been focused on growth kinetics and optimization of β-galactosidase production by thermophilic lactic acid bacteria, and most of them suggested reconstituted skim milk as the most effective medium, which is fairly expensive, and increases the risk of phage contamination during the enzyme production process [11,19,20].

Nowadays, availability of commercial bulk starter media could be advantageous for β-galactosidase production because for production of yoghurt or cheese thermophilic cultures are complex blend of skim milk powder or whey protein concentrate supplemented with nutrients, buffering and chelating agents and free of inhibitors so, they are specifically designed to support the propagation of these starter cultures [21]. This medium can be combined with external pH control to produce higher cell densities and enzyme concentrations along with reducing the probability of phage problems in the fermentation process.

This platform can provide a novel strategy for production of a cost-effective β-galactosidases source for industrial applications.

In this study, the effectiveness of two common commercial bulk starter media along with skim milk was compared for growth and β-galactosidase production using the kinetic parameters obtained from the growth, lactic acid production and enzyme activity models. In addition, β-galactosidase production was simulated in all three tested media based on growth and lactic acid production using different kinetic models.

## 2. Materials and Methods

### 2.1. Microorganisms and media

*Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081, a common yoghurt culture, was chosen for this study because of its well-known high β-galactosidase producing activity [22]. The strain was obtained from DSMZ culture collection (DSMZ, Germany) and stored at -80°C in 2 ml cryo-vials containing 50% broth culture medium, 25% (v $v^{-1}$) glycerol (G9012, Sigma-Alderich, USA) and 25% (v $v^{-1}$) skim milk (70166, Fluka, Germany) as cryo-protectant. Three media were used for fermentations: Media12®, a commercial medium based on skim milk and whey (Danisco A/S, Niebüll, Germany), designed for thermophilic cultures on external pH control, VIS-START® TW60, a commercial medium based on whey (Danisco A/S, Niebüll, Germany), designed for mesophilic and thermophilic cultures and reconstituted skim milk as a control medium.

### 2.2. Determination of nitrogen fractions, lactose and lactic acid concentration

The Kjeldahl method (K-350, Buchi, Switzerland) was used for determination of total nitrogen, total protein, and non-protein nitrogen content (NPN) of the three culture media [23]. Lactose and lactic acid concentration was determined by high performance liquid chromatography (HPLC) (K-1001, KNAUER, Berlin-Zehlendorf, Germany) equipped with a aminex column (Nucleosil 100-5$NH_2$, Hichrom Limited, UK) using a RI detector [24]. The deproteinized samples were eluted during 12 min under ACN/$H_2O$ (80% of acetonitrile, and 20% of double distilled water, v $v^{-1}$) at 0.8 ml $min^{-1}$. All the experiments were conducted in triplicate.

### 2.3. Fermentations

A 2-L stirred tank bioreactor (BIOFLO 2000, New Brunswick Scientific Co., New Brunswick, NJ, USA) was used for fermentation. After sterilization (121°C,15 min), the bioreactor was filled with 1940 ml of the heat treated media (95°C, 30 min) or sterilized skim milk. The media were inoculated with 60 ml of the inoculum prepared with the same medium to the fermentation medium incubated at 42-43°C for 10 h. Temperature was controlled at (43±1°C), agitation was controlled at 60 rpm, and pH was set at 5.6±0.01 by automatic addition of 6 N KOH.

### 2.4. Determination of growth parameters

Viable counts were enumerated as colony forming units (CFU $ml^{-1}$) by surface plating dilutions on the lactose-enriched MRS medium with agar (15 g $l^{-1}$, Difco) and incubating under microaerophilic condition (5% $CO_2$) at 43°C for 48 h. The total biomass concentration was determined by optical density measurements (Varian, Carry 300, Australia) at 600 nm, which was converted into biomass dry weight using a calibration curve. The rate of lactose uptake and lactic acid production was monitored by HPLC as described above. All trials were repeated tree times, and the results were reported as means and standard deviations.

### 2.5. β-galactosidase activity assay

The amount of o-nitrophenol (ONP) released from o-nitrophenol-β-D-galactopyranoside (ONPG, 73660, Sigma-Alidrich, USA) as chromogenic substrate was used to measure β-galactosidase activity based on the modified Miller;s method [25]. A 100-µl aliquot of the culture was added to 900 µl of Z buffer (0.06 M $Na_2HPO_4$; 0.04 M $NaH_2PO_4$; 0.01 M KCl; 0.001 M $MgSO_4$-7 $H_2O$). 100 µl of chloroform and 50 µl of 0.1% SDS were added, and the tubes were vortexed for 15 s, and incubated for 5 min at 28°C water bath. Afterwards, 200 µl of ONPG (4 mg $ml^{-1}$ in 0.1 *M* phosphate buffer) was added, and the mixture was incubated at 37°C for 15 min. The reaction was terminated by addition of 500 µl of 1 M $Na_2CO_3$. The samples were centrifuged at 10000 ×g for 5 min, and

then the absorbance was measured at 420 nm. The nano-moles of ONP liberated were determined from a standard curve measuring the change in absorbance produced by various ONP concentrations in the range of 200-1000 nmol. The amount of ONP released $min^{-1}$ is directly proportional to the quantity of enzyme and one unit of β-galactosidase activity (U $min^{-1}$ $ml^{-1}$) is defined as the amount of enzyme that hydrolyzes 1 nmol of ONPG to o-nitrophenol per minute at 37°C and pH=7. Enzyme activity was assayed every hour during the fermentation, and all trials were repeated three times.

### 2.6. Model description of batch Fermentation

#### 2.6.1. Growth kinetics

The modified logistic equation of Garcia-Ochoa and Cases [26] was used to model the growth kinetics. Garcia-Ochoa and Cases model is a simplified, unstructured, non-segregated, kinetic model, which was developed for the production of sophorolipids by *Candida bombicola*. This kinetic model assumes that the nitrogen is the limiting nutrient for yeast growth and sophorolipid production. This model was selected in this study because nitrogen is usually the limiting factor in lactic acid bacterial growth in skim milk and whey based media, when the carbohydrate source is not limiting [27]. The specific growth rate in this model is $\mu = (dX/dt)/(1-(X/X_m))$, and a correction factor ($k_c$) is used to avoid from uncertainty in the model data:

$$\frac{dX}{dt} = k_c\ \mu\left[1-\frac{X}{X_m}\right] \qquad \text{Eq. 1}$$

where, $X$ is the biomass concentration (g $l^{-1}$), $\mu$ is the specific growth rate ($h^{-1}$), and $X_m$ the maximum concentration of the biomass tested at the end of the fermentation. Integration from Eq. 1 with the initial condition of $X=X_0$ (cell concentration at the beginning of fermentation ($t$=0), gives a sigmoidal curve representing both exponential and stationary phases Eq. 2:

$$X(t) = \frac{X_0\ e^{(\mu k_c t)}}{1-\frac{X_0}{X_m}\left(1-e^{(\mu k_c t)}\right)} \qquad \text{Eq. 2}$$

#### 2.6.2. Lactic acid and β-galactosidase production kinetics

The Luedeking-Piret kinetic has been widely used for describing the relationship of cell growth to product formation [28]. We used this kinetic model for both lactic acid and β-galactosidase productions. Eq. 3 illustrates an empirical relationship between the rate of cell growth and product formation in growth and non-growth associated phases:

$$dP/dt = \alpha\, dX/dt + \beta X \qquad \text{Eq. 3}$$

Where, P is the product concentration. In this study lactic acid concentration (mg $ml^{-1}$) or β-galactosidase activity (U $min^{-1}$ $ml^{-1}$) and $\alpha$ and $\beta$ are empirical constants, which represent the growth and non-growth (maintenance) associated lactic acid and β-galactosidase production, respectively. Both coefficients are dependent upon the strain, growth medium and fermentation conditions [28]. The term $\alpha$ dX/dt is referred to as the product formation rate associated with growth, and describes the additional product formation by the growing organism. The term $\beta$X is the product formation rate associated with non-growth condition. The maintenance constant ($\beta$) was obtained from the stationary growth phase ($dX/dt = 0$), while the procedure of Luedeking and Piret [28] and Weiss and Ollis [29] was used for growth-associated constant ($\alpha$) determination using linearized experimental data in the exponential phase. Based on these, integrations from Eq. 3 were driven. The lactic acid and β-galactosidase production associated with the growth was estimated from:

$$P_\alpha = \frac{X_0\,(1-\frac{X_0}{X_m})\,(e^{k_c\,\mu t}-1)}{1-\frac{X_0}{X_m}(1-e^{k_c\,\mu t})} \qquad \text{Eq. 4}$$

And the non-growth associated lactic acid production was calculated by:

$$P_\beta = \beta\frac{X_m}{k_c\mu}\ln\left[1-\frac{X_0}{X_m}\,(1-e^{\,k_c\mu t})\right] \qquad \text{Eq. 5}$$

Parameters μ, α, β, $P_\alpha$ and $P_\beta$ were determined for each fermentation, and mean values and standard deviations were calculated from three culture replicates.

#### 2.6.3. β-galactosidase production kinetics based on lactic acid production

Since β-galactosidase is crucial enzyme for lactose metabolism and lactic acid production, its production could be correlated to lactic acid production [24]. Consequently, we assumed a direct relation between β-galactosidase production and lactic acid production rate as well as its amount in the fermentation medium. This was expressed with the following formula by analogy with the Luedeking-Piret equation:

$$dE/dt = \alpha dP/dt + \beta P \qquad \text{Eq. 6}$$

Where, P is the lactic acid concentration (mg $ml^{-1}$), E is the β-galactosidase activity (U $min^{-1}$ $ml^{-1}$), and $\alpha$ and $\beta$ are empirical constants. Structure of Eq. 6 is the same as the kinetic model [28]. Beginning of the logarithm phase is assumed as t=0. Integration of Eq. 6 with the initial conditions of $E=E_0$ (the enzyme concentration at t=0) and $P=P_0$ (the lactic acid concentration at t=0) gives Eq. 7:

$$E(t) = \alpha\,P(t) + \beta\int_0^t P(t)\,dt + (E_0 - \alpha P_0) \qquad \text{Eq. 7}$$

### 2.7. Statistical analysis

The maple software was used to solve the model equations, and treatment effects were compared by using Hsu's MCB test with Minitab 15.1.1.0 software. For simplicity of calculations, the trapezoidal method was employed to calculate the integration value of

second term in Eq. 4.

## 3. Results and discussion

### 3.1 Lactose and nitrogen content of the media

Table 1 shows some features of the three used bulk starter media. Compositional analysis showed that skim milk had the highest lactose, total nitrogen and protein content. However, the NPN content of two commercial bulk starter media was higher than skim milk. This higher NPN is related to supplementation of the commercial media with readily available nitrogen sources such as yeast extract, whey protein concentrate, and casein hydrolysate [30,31]. The maximum and minimum NPN content and NPN/Total nitrogen ratio were observed in Media12® medium and skim milk, respectively.

### 3.2 Growth kinetics

The biomass production and viable cell count profiles of *L. bulgaricus DSM 20081* growth in the commercial bulk starter media and skim milk are shown in Figure 1. Cultivation in skim milk resulted in higher biomass concentration (6.74 g $l^{-1}$) compared to the commercial bulk starter media (Figure 1A). However, the commercial bulk starter media growth cultures showed a rapid growth in the early stage of fermentation (no lag phase was detected), shorter exponential growth phase, and fast slow down after reaching to a maximum viable cell count compared to the skim milk growth culture. Based on the results of viable cell count, it was observed that the highest cell counts obtained in the skim milk and Media12® growth cultures were 9.78 and 9.65 log (CFU $ml^{-1}$), respectively (Figure 1B). These were significantly higher than the VIS-START® TW60 growth culture 9.46 log (CFU $ml^{-1}$) ($P$<0.05). The highest specific growth rate and maximum growth rate ($\mu_{max}$) were obtained in Media12® medium, which is based on skim milk and whey followed by VIS-START® TW60 based on whey (Table 2). These higher growth rates could be well correlated to supplementation of the commercial bulk starter media with readily available nitrogen sources and functional ingredients as well as favorable lactose concentration (Table 1). Vasiljevic and Jelen (2001) reported similar growth kinetics for the pH-controlled batch fermentation of *L. bulgaricus* in skim milk and whey supplemented with MRS broth, yeast extract, and whey protein concentrate. They observed higher growth rate and total cell count for whey supplemented with 1.2% MRS and skim milk [24,32]. As shown in Table 1, in spite of higher total nitrogen and protein content in the skim milk medium, the percentage of NPN and the ratio of NPN content to total nitrogen content were higher in the commercial bulk starter media, which resulted in higher activity of the culture. The positive effect of these components was also demonstrated for lactic acid fermentation by *L. rhamnosus* and *L. helveticus* [33,34]. The lactose concentration is other important factor in performance of lactic acid fermentation [32, 33]. The lower lactose concentration in the commercial bulk starter media could exert a positive effect on the growth rate (Figure 2). Burgos-Rubio et al. observed higher cell densities, decreased residence time, and higher global productivity for *L. bulgaricus* with the initial lactose concentration of 39 (g $l^{-1}$) and linear decay of the growth at higher lactose concentrations [35].

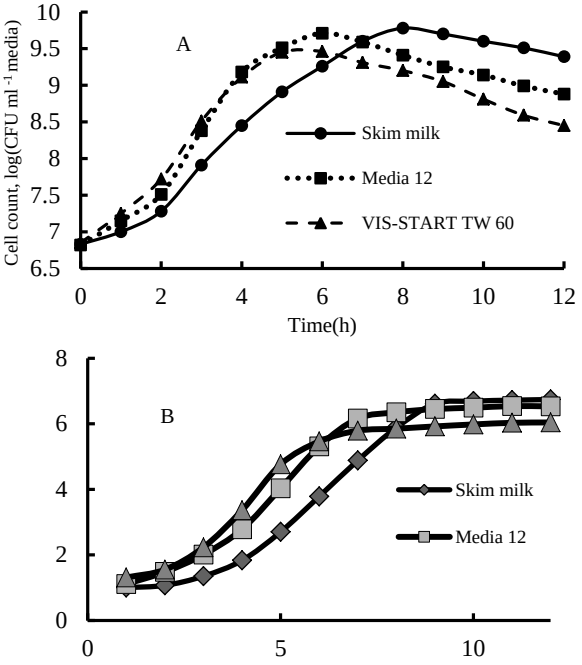


**Figure 1.** Viable cell count (A) and biomass production (B) of *L. bulgaricus* DSM 20081 growth in two commercial bulk starter media and skim milk at pH=5.6 and T=43°C (Error bars are not shown because their size is within the size of the symbols).

**Table 1.** Compositional data and features of two commercial bulk starter media and skim milk used for cultivation of *L. bulgaricus* DSM 20081 and β-galalctosidase production.

| Media | Total solids | pH | Lactose concentration(g $l^{-1}$) | Total nitrogen content (%) | Total protein content (%) | NPN (%) | $^{R2}$/Total nitrogen ratio |
|---|---|---|---|---|---|---|---|
| VIS-START®[1] | 6% | 6.15 | 35.3 ± 0.4$^{a}$ | 0.288± 0.003$^{a}$ | 1.85± 0.12$^{a}$ | 0.5± 0.04$^{b}$ | 1.73$^{b}$ |
| Media 12®[2] | 6.8% | 6.7 | 39.1 ± 0.3$^{b}$ | 0.412± 0.005$^{b}$ | 2.63± 0.11$^{b}$ | 0.89± 0.09$^{c}$ | 2.17$^{c}$ |
| Skim milk | 10% | 6.8 | 48.6 ± 0.6$^{c}$ | 0.511± 0.004$^{c}$ | 3.26± 0.14$^{c}$ | 0.16± 0.04$^{a}$ | 0.31$^{a}$ |

[1]Ingredients: Whey, Whey protein concentrates (WPC), casein hydrolysate, and minerals
[2] Ingredients: Skim milk powder, Whey powder, yeast extract, and minerals
Means with different letters (a-c) within a column are significantly different ($p$ < 0.05)

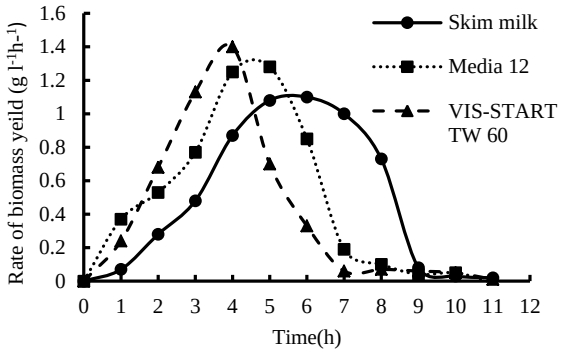


**Figure 2.** Specific growth rate of *L. bulgaricus* DSM 20081 growth in two commercial bulk starter media and skim milk at pH=5.6 and T=43°C (Error bars are not shown because their size is within the size of the symbols).

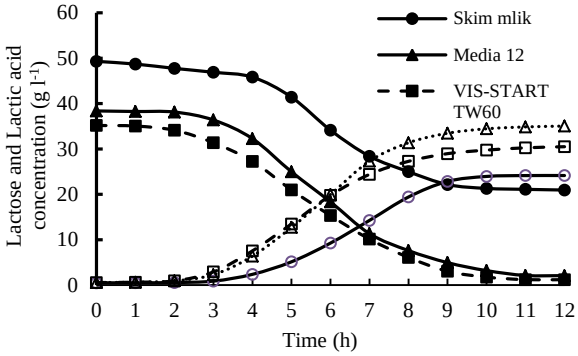


**Figure 4.** Lactose uptake (open symbols) and lactic acid production (closed symbols) kinetics of *L. bulgaricus* DSM 20081 cultivated in two commercial bulk starter media and skim milk at pH=5.6 and T=43°C (Error bars are not shown because their size is within the size of the symbols).

However, the lower total solids and substrate limiting effect are the most important predictable mechanism for the rapid growth cessation of the commercial bulk starter growth culture [36].

Figure 3 shows the simulations of cell growth based on biomass concentration with the modified model of Garcia-Ochoa and Cases with a correction factor (Eq. 2). The model was fitted well with the experimental data of growth kinetics in the exponential and stationary phases of *L. bulgaricus (*$R^2$ = 0.98) in all three studied media. However, the correlation coefficients obtained for simulated and experimental data of viable cell counts were not satisfactory for MEDIA12® and VIS-START® TW60 media using the same model. This poor correlation could be related to the sudden growth cessation of the commercial bulk starter growth culture after reaching to maximum cell count (data not shown).

### 3.3. Lactic acid production kinetics

Cultivation of *L. bulgaricus* DSM 20081 in the commercial bulk starter medium resulted in higher lactose conversion and lactic acid production comparing to skim milk (Figure 4).

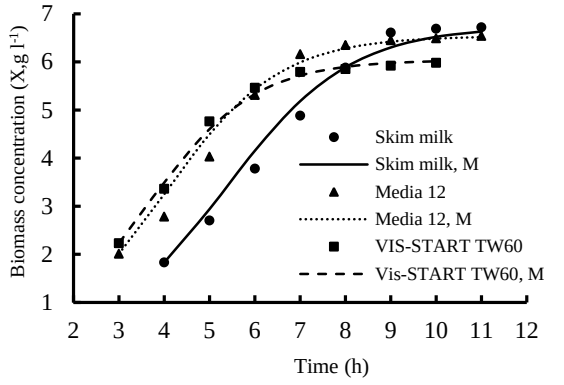


**Figure 3.** Simulation of biomass yield (g $l^{-1}$) during the batch fermentation of *L. bulgaricus* DSM 20081 in two commercial bulk starter media and skim milk at pH=5.6 and T=43°C with modified Garcia method (Eq. 2). Experimental data; symbols (● Skim milk, ▲ Media 12®, ■ VIS-START® TW60), model simulations (Continuous lines).

As shown in the figure, the commercial bulk starter growth cultures have approximately consumed all lactose content of the media while the skim milk growth culture consumed about 40% of the lactose content. The maximum and minimum total lactic acid production was obtained in Media12® medium (35.01 g $l^{-1}$) and skim milk medium (24.14 g $l^{-1}$), respectively. Similar results were observed for the rate of lactic acid production (Figure 5). The pattern for lactic acid production rate was similar to the specific growth rate profile (Figure 2). The rate of lactic acid production in the commercial bulk starter medium was higher than in skim milk.

The maximum rate of lactic acid production was obtained in Media12® (7.3 mg lactic acid $ml^{-1}$ $h^{-1}$) after 7h, which differed significantly ($P$<0.05) from the skim milk growth culture (4.56 mg lactic acid $ml^{-1}$ $h^{-1}$, after 8h). This higher lactic acid production could be correlated to higher activity of the culture and the positive effect of medium supplementation with the readily available nitrogen sources, as described for the cell growth and biomass production. The functionality of these nitrogen sources were well demonstrated for improving lactic acid production by lactic acid bacteria such as *L. bulgaricus* and *L. helveticus* [37-39]. Kinetic parameters of lactic acid production using descriptive lactic acid production models are presented in Table 2. The higher ratio of $P_\alpha/P_\beta$ in the commercial bulk starter medium shows higher lactic acid production as well as the growth associated lactic acid production mechanism in all the three studied media. The lower maintenance related parameter of lactic acid production in the commercial bulk starter media is associated with the higher growth rate during the exponential phase, which resulted in nutrition deficiency during the stationary phase [40]. The model simulation and experimental data for lactic acid production in the commercial bulk starter media and skim milk are presented in Figure 6. The proposed lactic acid production model of Luedeking-Piret showed good agreement with the experimental data over the exponential and stationary growth phases of *L. bulgaricus* DSM 20081 in all the three tested media. The correlation coefficients ($R^2$) for all the three culture media were over 0.98.

**Table 2.** Kinetic parameters of the growth and lactic acid production for *L. bulgaricus* DSM 20081 in two commercial bulk starter media and skim milk (mean of three replicates).

| Medium | Specific growth rate, $h^{-1}$ | $\mu_{max}$ ($g\ l^{-1}$) | $\alpha^*$ | $\beta^*$ ($t^{-1}$) | $P_\alpha^{**}$ | $P_\beta^{**}$ | $P_\alpha/P_\beta$ | Maximum rate of acid production, mg $ml^{-1}$ $h^{-1}$ | Peak time for acid production (h) |
|---|---|---|---|---|---|---|---|---|---|
| VIS-START® | $0.73 \pm 0.03^{c}$ | $1.06 \pm 0.02^{b}$ | 4.19 | 0.23 | $16.65^{c}$ | $8.10^{c}$ | $2.04^{b}$ | $6.1 \pm 0.15^{b}$, after 6h | 6 |
| Media 12® | $0.77 \pm 0.04^{b}$ | $1.17 \pm 0.03^{c}$ | 5.24 | 0.21 | $23.70^{a}$ | $10.2^{b}$ | $2.32^{c}$ | $7.3 \pm 0.10^{c}$, after 7h | 7 |
| Skim milk | $0.62 \pm 0.02^{a}$ | $0.96 \pm 0.01^{a}$ | 3.10 | 0.17 | $16.53^{b}$ | $10.9^{a}$ | $1.52^{a}$ | $5.1 \pm 0.20^{a}$, after 8h | 8 |

* α and β-coefficients associated with growth and maintenance-related lactic acid production, respectively.
** $P_\alpha$ and $P_\beta$ -growth and maintenance associated lactic acid production calculated from Eq. 4 and Eq. 5, respectively.
Means with different letters (a-c) within a column are significantly different ($p < 0.05$).

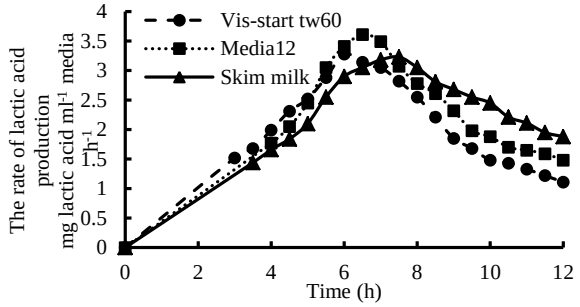


**Figure 5.** Rate of lactic acid production of *L. bulgaricus* DSM 20081 cultivated in two commercial bulk starter media and skim milk at 43°C, pH=5.6 (Error bars are not shown because their size is within the size of the symbols).

### 3.4. β -galactosidase activity

To investigate the effectiveness of bulk starter fermentation strategy for β-galactosidase production, β-galactosidase activity profiles and production kinetic parameters were compared in the commercial bulk starter media and skim milk. Figure 7 shows the β-galactosidase activity kinetics of *L. bulgaricus* DSM 20081 in different media. The maximum β-galactosidase activity in Media12® growth culture reached to 801 (nmol ONP released $ml^{-1}$ media) after 8 h which was significantly higher than for skim milk and VIS-START® TW60, with 767 (nmol ONP released $ml^{-1}$ media) and 622 (nmol ONP released $ml^{-1}$ media) after 9 and 7 h, respectively ($P<0.05$). Table 3 shows the kinetic parameters of β-galactosidase production of *L. bulgaricus* DSM 20081 in three studied media. As could be seen, the growth related production of β-galactosidase ($P_\alpha/P_\beta$) was the highest for Media12®. In contrast, the negative values for the non-growth production parameter ($P_\beta$) could be related to lower expression and/or loss of enzymatic activity during the stationary and decline growth phases. Similar results were also reported for production of β-galactosidase by thermophilic lactic acid bacteria in skim milk, whey and MRS based media [11,19]. Thus, the higher β-galactosidase activity in Media 12® could be well correlated to higher growth and lactic acid production rate in this medium in compare to the two other media. Furthermore, β-galactosidase activity was also shown to be dependent on the media components such as carbon and nitrogen source as well as the buffering capacity of the media, which are more favorable in Media12®.

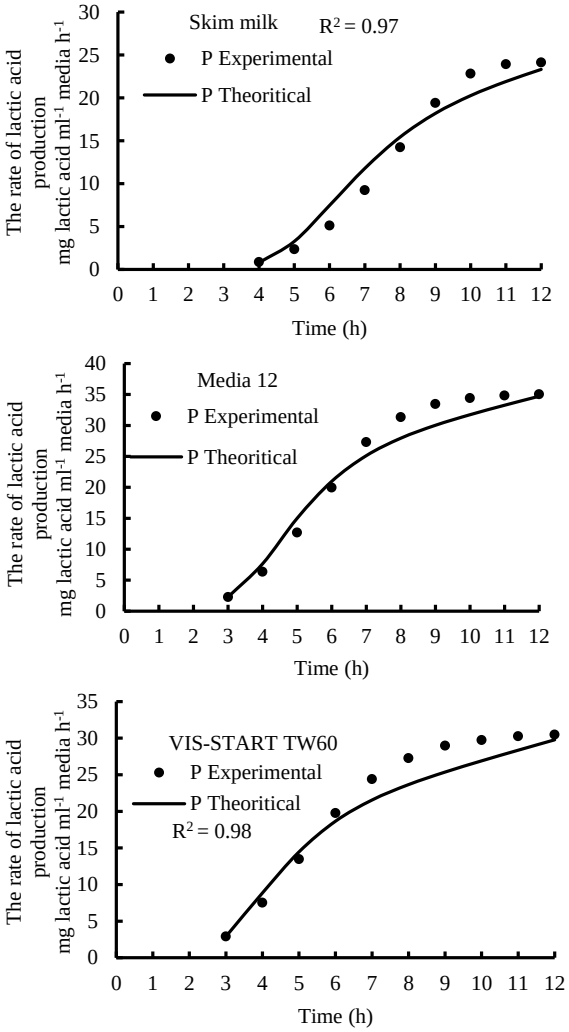


**Figure 6.** Comparison between model simulation (using Luedeking-Pieret method (Eq. 3)) and experimental data for lactic acid production in the batch culture of *L. bulgaricus* DSM 20081 in two commercial bulk starter media and skim milk at pH=5.6, T=43°C. Experimental data; symbols (● Skim milk, ▲ Media12®, ■ VIS-START® TW60), model simulations (Continuous lines).

Simulations of β-galactosidase activity based on biomass production using Luedeking-Piret method (Eq. 4) are shown in Figure 8. A good agreement between the calculated and experimental values was observed for skim milk ($R^2$=0.99) and Media12® ($R^2$=0.97). However, simulation results for VIS-START® TW60 medium were not satisfactory ($R^2$=0.93) using the same method (Eq. 4).

**Table 3.** Kinetic parameters of β-galactosidase production of *L. bulgaricus* DSM 20081 in two commercial bulk starter media and skim milk.

| Medium | Maximum β-galactosidase activity (nmol ONP $ml^{-1}$ media) | Residence time (h) | α (nmol onp $mg^{-1}$) | β | $P_\alpha$ | $P_\beta$ | $P_\alpha/P_\beta$ |
|---|---|---|---|---|---|---|---|
| VIS-START® | 612.2 ± 0.2 | 7 | -1.96 | 113.2 | 430.94[a] | -92.68[c] | -4.651 |
| Media 12® | 800.1 ± 0.7 | 8 | -3.13 | 152.1 | 687.54[c] | -154.09[b] | -4.464 |
| Skim milk | 777.1 ± 0.2 | 9 | -7.03 | 135.3 | 657.4[b] | -293.68[a] | -2.238 |

* α and β-coefficients associated with growth and maintenance-related β-galactosidase production, respectively.
** $P_\alpha$ and $P_\beta$ -growth and maintenance associated
β-galactosidase production calculated from Eq. 4 and Eq. 5, respectively.
Means with different letters (a-c) within a column are significantly different ($p < 0.05$).

To obtain better simulations, β-galactosidase production kinetics was simulated based on lactic acid production using Eq. 7. As shown in Figure 9, simulation based on lactic acid production resulted in a good estimation for skim milk, Media12®, and VIS-START® TW60 media with the correlation coefficients ($R^2$) of 0.99, 0.98, and 0.96, respectively. These results show the advantage of simulation of enzymatic activity based on the rate of lactic acid production, which was not previously reported. These good correlations along with similar patterns observed for lactic acid and β-galactosidase production could be advantageous for indirect determination of β-galactosidase activity through lactic acid production using the proposed model.

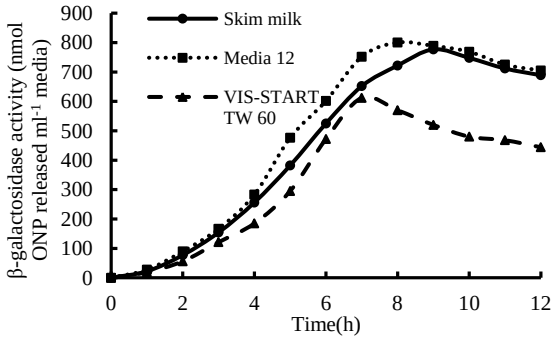


**Figure 7.** β-galactosidase activity of *L. bulgaricus* DSM 20081 cultivated in the two commercial bulk starter media and skim milk (Error bars are not shown because their size is within the size of the symbols).

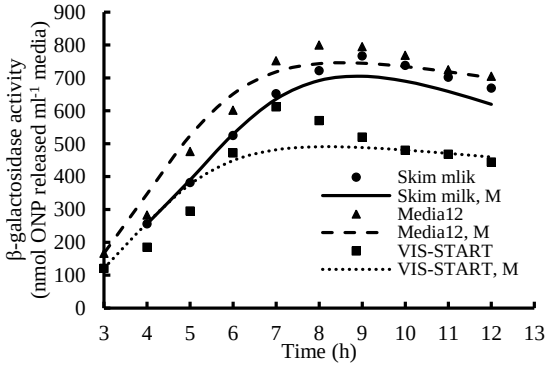


**Figure 8.** Comparison between model simulation and experimental data for β-galactosidase activity in the batch culture of *L. bulgaricus* DSM 20081 in two commercial bulk starter media and skim milk at pH=5.6, T=243°C. Experimental data; symbols (● Skim milk, ▲ Media 12®, ■ VIS-START® TW60), model simulations (Continuous lines).

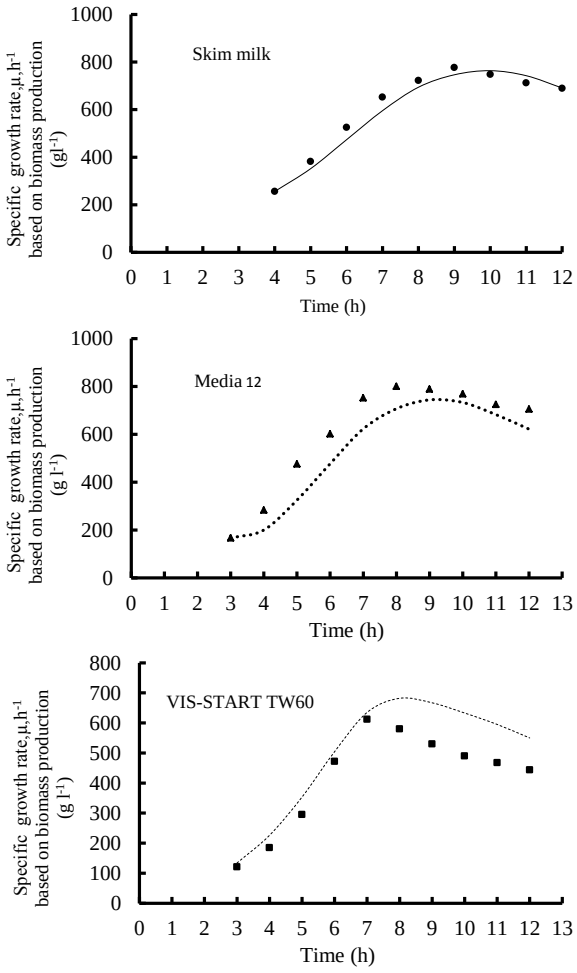


**Figure 9.** Model simulation of β-galactosidase production kinetics based on lactic acid production in the batch culture of *L. bulgaricus* DSM 20081 in two commercial bulk starter media and skim milk at pH=5.6, T=43°C using Eq. 7 . Experimental data (● Skim milk, ▲ Media 12®, ■ VIS-START® TW60), model simulations (Continuous lines).

## 4. Conclusions

Results presented in this paper clearly show the advantage of commercial bulk starter medium based on skim milk and whey (Media12®) for β-galactosidase production from thermophilic starter cultures. This medium supports better growth, lactic acid production rate, and enzyme synthesis by *L. bulgaricus* in pH-controlled batch fermentation compared to skim milk, which is common bulk starter medium in yoghurt and cheese production. The higher β-galactosidase activity in Media 12® could be well

correlated to its favorable lactose content, higher percentage of NPN and improved buffering capacity due to addition of buffering salts. Improved and more consistent fermentation, reduced fermentation time, and application dependent but often reduced cost-in-use of fermentation are among the main advantages of commercial bulk starter media over reconstituted skim milk in enzyme production. Therefore, besides exploring suitable medium, the bulk starter fermentation strategy could be easily adopted for in-house production of β-galactosidase from thermophilic lactic acid bacteria for lactose hydrolysis and galacto-oligosaccharide production applications by small dairy plants.

Finally, a model was developed for in-direct estimation of β-galactosidase activity using lactic acid production rates, which could accurately describe the β-galactosidase activity profile and also deceleration of enzyme activity in all the three culture media. Also it can be concluded that using modified Garcia and Luedeking-Piret models, biomass yield, lactic acid production and the end-point of fermentation can be conveniently estimated for maximizing β-galactosidase production.

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## 6. Conflict of interest

The authors declare no conflict of interests.

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