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Production of β-galactosidase by *Streptococcus salivarius* subsp *thermophilus* 11F

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The production of β-galactosidase by an autolytic strain of *Streptococcus salivarius* subsp *thermophilus* 11F was investigated in batch and fed-batch 2-L working volume stirred tank bioreactors. β-Galactosidase was released into the medium upon cell lysis within 1–2 h after the maximum biomass quantity was reached. In batch fermentations the highest β-galactosidase activity of 69 U ml^{-1} was obtained when the temperature was increased to 42°C after a 4-h growth period at 30°C. In fed-batch experiments the highest β-galactosidase activity of 74 U ml^{-1} was obtained at a constant 37°C.

Keywords: enzyme production; fed-batch fermentation; β-galactosidase; lactase; *Streptococcus salivarius* subsp *thermophilus*

Introduction

A food grade thermostable β-galactosidase (lactase, β-d-galactosidase galactohydrolase, EC 3.2.1.23) with a neutral pH-optimum would be invaluable in a number of food industry applications. Nutritional and technological benefits of partial enzymic hydrolysis of lactose in milk, whey, and other dairy products have been clearly demonstrated [2,3,10,16], and continuous, commercial scale hydrolysis of cheese whey lactose by immobilized mold *Aspergillus niger* β-galactosidase has been in operation since 1978 [6]. Another novel possibility is the production of oligosaccharides by utilizing the transferase activity of β-galactosidase [1,7,9,13]. While some bacterial sources for β-galactosidase have been identified, either poor yield or a requirement for safety clearance has been the bottleneck for commercial use [5,20]. The intracellular β-galactosidase of the yeast *Kluyveromyces fragilis* is relatively unstable under processing conditions [21] but could be used either as immobilized [19] or as free soluble [4] enzyme for the treatment of milk.

Ramana Rao and Dutta [11] demonstrated already in 1977 that *Streptococcus salivarius* subsp *thermophilus* (in the following: *S. thermophilus*) produces relatively high quantities of intracellular β-galactosidase. Somkuti and coworkers [17,18] showed that *S. thermophilus* cells could be permeabilized by a number of compounds of detergent activity resulting in high-level expression of the intracellular enzyme. The permeabilized cells could then be used directly for the hydrolysis of lactose in milk. Sandholm and Sarimo [12] showed that certain autolytic *S. thermophilus* strains release the intracellular β-galactosidase to the medium upon cell autolysis, which would markedly simplify downstream processing in the production of the enzyme. The enzyme is relatively thermostable, and it has been purified and characterized [2,15]. Further, *S. thermophilus* is a GRAS-organism widely used in the production of fermented dairy foods and, consequently, is an ideal organism for food enzyme production. The present paper demonstrates that high β-galactosidase activities can be released to the medium in fed-batch fermentations of the autolytic *S. thermophilus* 11F using conventional stirred tank bioreactors, and that the end point of the fermentation can be conveniently estimated by a relatively simple neural network.

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Materials and methods

Bacterial strain

Streptococcus thermophilus 11F was obtained from the Valio Laboratory (Helsinki, Finland). The organism was maintained on glass beads at –80°C and revived from the lyophilized state in autoclaved skim milk by growing for 17 h at 42°C, using a single bead. The quality of the inoculum was followed using °SH-degree. One °SH was defined as the amount of 0.25 M NaOH solution needed to neutralize 100 ml of milk.

Production media

The whey permeate-based production medium contained either 0.25% casein hydrolysate (Valio) or 1% proteose peptone (Difco), 0.25–1% yeast extract (Difco), and 6% deproteinized whey (Valio). In some cases 0.1% Tween 80 was added to the medium. Each component was autoclaved separately for 15 min at 121°C and combined aseptically before transferring to the fermentor with a peristaltic pump, as it has been shown previously that β-galactosidase production is very sensitive to the degree of heat treatment of the medium.

Enzyme production

Fermentations were carried out in 3-L (2-L working volume) Biostat® M or 10-L working volume Biostat® E

fermentors (B Braun Melsungen, Germany). The inoculum was prepared by growing the bacteria in 10% skim milk for about 10 h at 42°C. After the addition of the inoculum (1% v/v), the cultures were stirred at 100 or 200 rpm without aeration under varying temperatures and temperature profiles, and the pH was controlled by automatic addition of ammonia. In fed-batch fermentations 300 ml of a medium containing 0.5% proteose peptone, 0.5% yeast extract (Difco), and 3% deproteinized whey was added at a time the maximum turbidity had been reached.

Neural networks for prediction

The neural network program used in the present work was written in Microsoft Visual C++ for Windows essentially as described by Linko *et al* [8] and implemented in a personal computer. The program was developed with a sufficient flexibility for easy parameter modification and data handling, and to provide a user-friendly graphic interface within MS-Windows.

Analytical methods

The increase in total cell mass, and cell lysis were followed spectrophotometrically (L = 600 nm). Light microscopy was used to obtain more detailed information on autolysis (nigrosin) and to investigate possible contamination of the cultures (methylene blue). Lactose was analyzed enzymically with a Boehringer Mannheim kit No. 176 303. β-Galactosidase was assayed at 45°C with lactose as a substrate by measuring the amount of glucose released from 5.55% (w/v) lactose in 0.1 M potassium phosphate buffer (pH 7.0). The reaction was terminated by the addition of perchloric acid to a 0.7% final concentration. After 3 h at room temperature, glucose was determined enzymically using the Boehringer Mannheim kit No. 124 036. One unit of β-galactosidase activity was defined as the amount of enzyme which releases 1 mmol glucose per minute.

Results and discussion

Batch process

The effect of temperature on *Streptococcus thermophilus* 11F growth, autolysis and β-galactosidase production was studied in 2-L working volume Biostat® M fermentors under different temperature profiles using a medium with 1% proteose peptone, 1% yeast extract and 0.01% Tween 80. The pH was maintained at 6.5 by automatic addition of 4 M ammonia, and the stirring speed was 200 rpm. In batch fermentations, little growth took place at 30°C. Fastest growth was obtained at a constant temperature of 42°C. If the microorganism was first maintained at 30°C for varying periods, growth was delayed until the temperature was increased to 42°C (Figure 1a). After reaching of the maximum turbidity characteristic of 42°C growth temperature, cells lysed normally regardless of the length of the holding period at 30°C. The maximum turbidity was obtained at a constant 37°C temperature, and at a constant 45°C both growth and enzyme production slowed down. However, if the microorganism was first grown for 4 h at 30°C followed by a temperature shift to 42 or 45°C, cells lysed normally and high β-galactosidase activity was obtained (Figure 1b, Table 1).

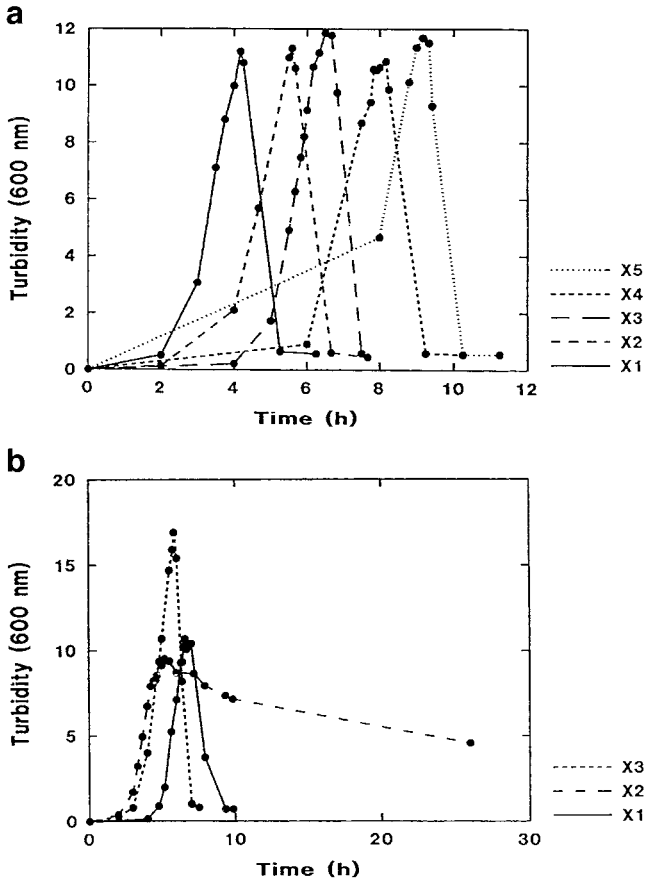

Figure 1 The effect of temperature on growth of *S. thermophilus* 11F: (a) 42°C (X_1), 2 h 30°C→42°C (X_2), 4 h 30°C→42°C (X_3), 6 h 30°C→42°C (X_4), 8 h 30°C→42°C (X_5); (b) 4 h 30°C→45°C (X_1), 45°C (X_2), 37°C (X_3).

Only about 45–68% of the lactose in the medium was utilized by *S. thermophilus* 11F under the experimental conditions used (Figure 2). According to Sandholm and Sarimo [12], high cell mass and degree of autolysis are obtained at 40–45°C, growth rate decreases at 30–37°C, and little autolysis takes place at 30°C.

The highest β-galactosidase activity of 69 U ml^{-1} in a batch fermentation was obtained, when the medium components were sterilized separately under a relatively mild heat treatment, and when the temperature during the fermen-

Table 1 Batch production of *Streptococcus thermophilus* 11F β-galactosidase in a Biostat® M fermentor

Temperature profile	Turbidity (600 nm)	β-Galactosidase activity (U ml^{-1})
37°C	16.9 (5.8 h)	60 (7.5 h)
42°C	11.2 (4.2 h)	64 (6.3 h)
2 h 30°C→42°C	11.3 (5.5 h)	58 (7.7 h)
4 h 30°C→42°C	**11.9 (6.5 h)**	**69 (7.5 h)**
4 h 30°C→45°C	10.7 (6.5 h)	59 (9.8 h)
6 h 30°C→42°C	10.8 (8.2 h)	63 (10.3 h)
8 h 30°C→42°C	11.7 (9.2 h)	47 (11.3 h)
45°C	9.5 (5.3 h) 4.6 (26 h)	19 (26.0 h)

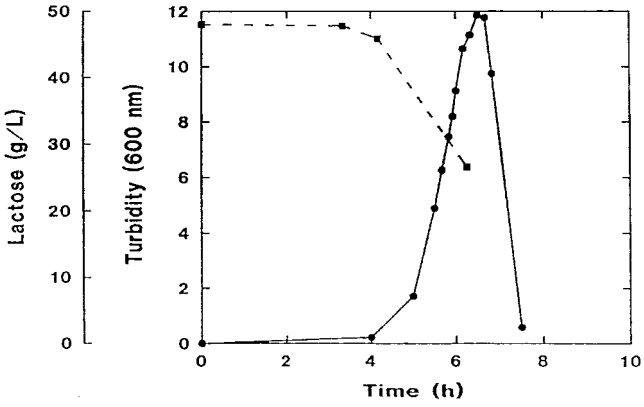

Figure 2 Turbidity (—) and lactose consumption (----) during the growth phase of *S. thermophilus* 11F in 2-L batch culture (4 h 30°C→42°C).

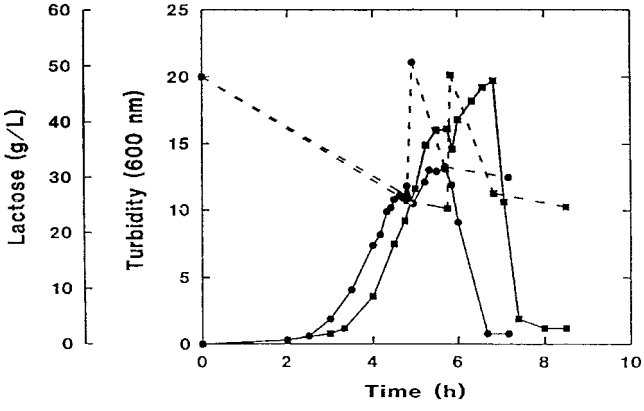

Figure 3 Turbidity (—) and the consumption of lactose (----) by autolytic *Streptococcus thermophilus* 11F in fed-batch fermentation at 37°C (■) and 42°C (●).

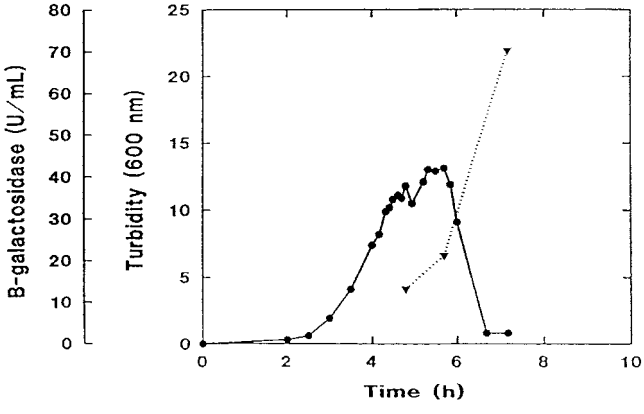

Figure 4 Turbidity (—) and β-galactosidase production (…) by the autolytic *Streptococcus thermophilus* 11F in a fed-batch cultivation at 42°C.

tation was increased after a 4-h growth period from 30 to 42°C (Table 1). With the release of the intracellular enzyme to the medium upon cell lysis, the β-galactosidase activity in the medium increased until about 2 h after the exponential growth phase ended. After that the β-galactosidase activity remained quite constant. If the temperature was shifted from 30 to 45°C, slightly lower β-galactosidase activity of 59 U ml^{-1} was obtained. At a constant 45°C,

Table 2 Fed-batch production of *Streptococcus thermophilus* 11F β-galactosidase in a Biostat® M fermentor

Cultivation temperature (°C)	Turbidity (600 nm)	Cell count (ml^{-1})	β-Galactosidase activity (U ml^{-1})
37°C[a]	**19.7 (6.8 h)**	–	**74**
42°C[a]	13.1 (5.7 h)	6.3×10^9	64
37°C[b]	10.6 (7.4 h)	2.9×10^9	58

[a]The medium contained 6% whey permeate, 1% proteoase peptone, 1% yeast extract, and 0.1% Tween 80.
[b]The medium contained 6% whey permeate, 0.25% casein hydrolysate, and 0.25% yeast extract.

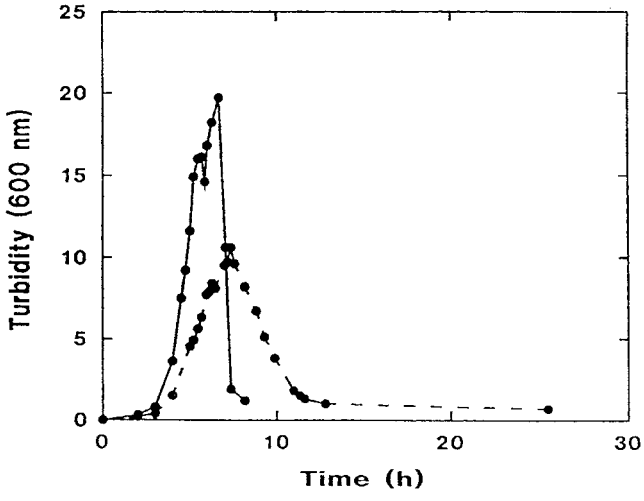

Figure 5 The effect of the type of medium on growth of the autolytic *Streptococcus thermophilus* 11F in a fed-batch cultivation at 37° (—, standard medium; ----, inexpensive medium).

cell autolysis was markedly retarded, the turbidity was still as high as 4.6 after 26 h, indicating incomplete cell lysis and, consequently, the activity of β-galactosidase released into the medium was at that time only 19 U ml^{-1}. It should be noted that if the complete medium was sterilized in the fermentation vessel for 40 min at 121°C, the maximum β-galactosidase activity obtained was only 4 U ml^{-1}. When the time of sterilization was decreased to 20 min, β-galactosidase activity more than doubled to 10 U ml^{-1} but still remained low as compared with the experiments in which the medium components were sterilized separately.

With the same strain, Smart *et al* [14] obtained *ca* 15 U ml^{-1} of β-galactosidase on a whey permeate-based medium in a 500-ml working volume fermentor at constant 42°C. However, they used a much higher agitation rate of 500 rpm, and maintained anaerobic conditions by continuous sparging with a mixture of nitrogen and carbon dioxide (95:5). Later, Smart and Richardson [15] reported the highest β-galactosidase activity of 19.9 U ml^{-1} on a synthetic medium. Also Chang and Mahoney [2] used a synthetic medium in the production of β-galactosidase by *S. thermophilus* 11F. In a 10-L culture, an enzyme activity of *ca* 5 U ml^{-1} was obtained after 7 h incubation at 42°C. In this case the shaking speed was 150 rpm.

Table 3 Reported *Streptococcus thermophilus* β-galacotosidase activities

Cultivation method	Working volume (ml)	Enzyme activity (U ml^{-1})	Remarks	Ref.
Stationary	Ng[a]	18.2	Strain 1, 40°C, pH 4.5–7, deproteinized whey, 2% proteose peptone, 24 h	[21]
Agitated, submerged	Ng[a]	11.3	Strain B-3641, 37°C, pH 5.3–7 2% deprot. whey, 2% proteose peptone, 3% CSL[b], 2% potassium phosphate buffer, 24 h	[1]
Stirred tank	500	>15	Strain 11F, 42°C, pH 6, 500 rpm, cont. sparging of N_2/CO_2 (95/5), 2.5% whey permeate, 0.225% polypeptone, 75 mM potassium phosphate buffer, 7 h	[14]
Stirred tank	1000	19.9	Strain 11F, 42°C, pH 6.8 cont. sparging of N_2/CO_2, J8 broth[c] + 2% lactose, 7 h	[15]
Stirred tank	2000	74	Strain 11F, fed-batch, pH 6.5, 200 rpm, 6% whey permeate, 1% proteose peptone, 1% yeast extract, 0.1% Tween 80, 7 h	present work
Stirred tank	2000	40	Strain 11F, fed-batch, pH 6.5, 200 rpm, 6% whey permeate, 0.25% casein hydrolysate, 0.25% yeast extract, 7 h	present work
Stirred tank	10000	5	Strain 11F, 42°C, pH 6, 150 rpm, J8 broth[c] + 2% lactose, 7 h	[2]

[a]Not given.
[b]Corn steep liquor.
[c]0.2% each of beef extract, yeast extract and phytone peptone, 0.5% poly peptone, 0.55% Na_2HPO_4, 0.5% KH_2PO_4, 0.2% $MgCl_2 \cdot H_2O$, and 0.05% ascorbic acid.

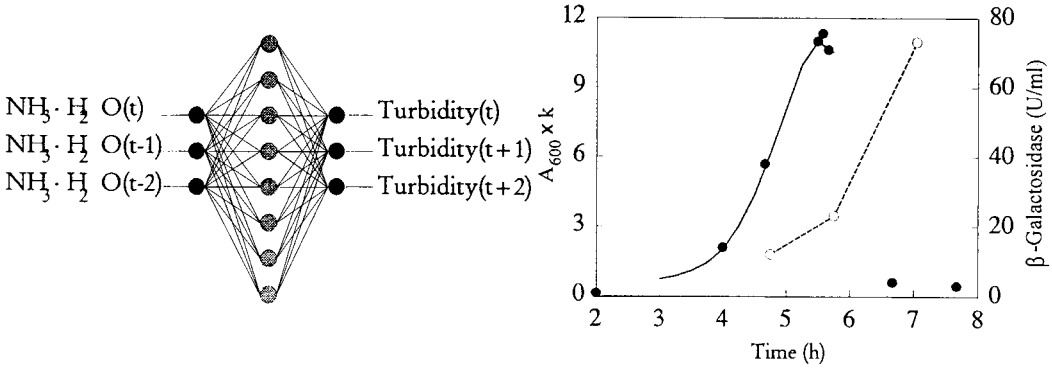

Figure 6 Neural network (left) used in one-step ahead prediction of biomass in β-galactosidase production (right) (●, measured and ——, estimated turbidity; ○, β-galactosidase activity; the shaded area represents the area for maximum β-galactosidase production).

Fed-batch process

In order to increase the total cell mass for an improved β-galactosidase activity in the supernatant after cell lysis, fed batch operation at a constant temperature was also investigated. In the fed-batch fermentations, additional nutrients were supplied to the fermentor at the time ammonia consumption slowed down and turbidity had reached its maximum value in about 5–6 h. A higher turbidity and, thus, total biomass, was indeed obtained in the fed-batch fermentations both at 37 and 42°C with a medium containing 1% proteose peptone, 1% yeast extract and 0.1% Tween 80 as compared to the conventional batch fermentations. β-Galactosidase activity did not, however, increase in the same order of magnitude. Typically, about 24–26 g ml^{-1} of lactose remained unused both at 37°C and at 42°C after the initial phase of about 5 or 6 h, respectively. After supplying additional nutrients, again 24 (37°C) to 30 g ml^{-1} (42°C) of residual lactose was left by the time the fermentation was discontinued after about 7 (42°C) or 8 (37°C) h (Figure 3).

Figure 4 illustrates the turbidity and released β-galactosidase activity as a function of time at constant 42°C. The autolysis began already well ahead of the time the maximum turbidity and biomass quantity was reached, but the autolysis could be delayed by the addition of nutrients. That some autolysis took place already during the exponential growth phase could be verified by microscopical observation. The highest β-galactosidase activity of 74 U ml^{-1} in the fed-batch experiments was, however, obtained at a constant temperature of 37°C (Table 2). Consequently, in a fed-batch mode β-galactosidase activity

obtained was higher even at a constant 37°C than in a batch mode involving an upward temperature shift. Further, the maximum activity obtained was almost four times higher than the activities reported earlier for *S. thermophilus* 11F [15,21].

In an attempt to reduce the medium costs, a modified, less expensive medium in which the quantity of yeast extract was decreased from 1% to 0.25% and proteose peptone was replaced by 0.25% casein hydrolysate was also tested under the otherwise above experimental conditions at a constant temperature of 37°C. Unfortunately, the use of the less-costly medium resulted in a much slower growth and a decreased rate of autolysis, and the maximum turbidity obtained was only about half of that obtained with the standard medium used in the fed-batch fermentations (Figure 5). Nevertheless, the β-galactosidase activities obtained with both media were markedly higher than the activities reported earlier by others for *S. thermophilus* β-galactosidase (Table 3).

Prediction of the end-point of fermentation

In industrial fermentations the ability of accurate advance prediction of the end point can be of great economic importance. Figure 6 shows example testing results in batch β-galactosidase fermentation with a well trained neural network of a relatively simple 3-8-3 topology, with ammonia consumption at times t, $t-1$ and $t-2$ as the inputs and turbidity at 600 nm at times t, $t+1$ and $t+2$ as the outputs (t = 15 min), for the prediction of the maximum turbidity two time intervals ahead. As the time interval was 15 min, the neural network estimator allowed an easy and accurate prediction of the maximum turbidity, for about 30 min in advance. As the maximum β-galactosidase activity in the medium appeared about 25 min after the maximum biomass concentration was reached as indicated by turbidity this, in turn, made it possible to predict the end point of the fermentation and to give ample warning in time.

Conclusions

The autolytic *Streptococcus thermophilus* 11F grew well under optimal conditions, autolysed rapidly at the end of the growth period, and released high quantities of β-galactosidase into the medium. The separate autoclaving of the medium components was essential for maximal growth β-galactosidase production. No contaminations were observed in spite of the relatively mild heat treatment. The highest β-galactosidase activity, 74 U ml^{-1} (almost 4-fold in comparison to the activities, that had been reported earlier for *S. thermophilus*), was obtained when the process was operated in a fed-batch mode at 37°C, pH 6.5, 200 rpm. High β-galactosidase activities were also obtained in batch fermentations, especially if the temperature was raised to 42°C after a short initial growth period at a constant 30°C.

Acknowledgements

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