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BIODEGRADATION OF WHEY WASTE IN A CONTINUOUS STIRRED-TANK BIOREACTOR

Post-production whey has a high concentration of proteins and lactose that has to be decreased 500-fold before disposal in the environment. The paper describes goat whey biodegradation in a continuous stirred tank reactor (CSTR) using a *Bacillus licheniformis* strain. The data obtained emphasize the effect of excess organic compounds on growth rate. Accordingly, the Luong equation was applied to describe the batch process while accounting for substrate inhibition; the constants $v_{max} = 2.29 \cdot 10^{-4}$ 1/s, $K_m = 4.79$ g/dm^3, $K_i = 6.03$ g/dm^3, and $n = 1.1$ were experimentally obtained. According to accepted theory, the continuous process can be expressed using Monod's equation (only for the rising side of the kinetic graph) in a restricted range of substrate concentrations (for glucose, up to 2.16 g/dm^3). The estimated values of the constants were $v_{max} = 8.52 \cdot 10^{-5}$ 1/s and $K_m = 0.45$ g/dm^3. The selected strain effectively decreased the lactose content in whey. At residence times above 30 h, almost total decrease in lactose content was observed that met the requirements of the Polish Ministry of Environmental Protection. The biodegradation of proteins was much slower, but their content does not greatly influence the required BOD and COD values.

1. INTRODUCTION

Whey is a by-product from the dairy industry. It is a liquid containing large amounts of lactose (39–45 g/dm^3) and proteins (9–14 g/dm^3). These high values result in high COD and BOD_5 coefficients of 60–80 g/dm^3 and 30–50 g/dm^3, respectively [1]. These high values are primarily due to the presence of lactose, as the recovery of proteins decreases the COD index approximately by 10 g/dm^3 [2]. Based on actual Polish Ministry of Environmental Regulation from 2014, whey is categorized as an extremely dangerous wastewater for aquatic environments, and its COD index should be reduced to 125 mg O_2/dm^3 and its BOD index to 25 mg O_2/dm^3.

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Whey is formed as a by-product on a large scale. The global production of whey is as high as 160×10^6 tons/year [3]. Only half of the world's whey production is consumed: 45% is used as a liquid in beverage production, 30% is dried and used in powder form in the food industry and agriculture, 15% is used as a source of lactose (its recovery is very expensive) [4], and the remaining 10% is used to produce whey protein concentrate (WPC) and whey protein isolate (WPI) [5]. The other 50% of produced whey is a serious problem for the dairy industry and has to be bioremediated.

However, whey proteins are valuable and have health-promoting properties; therefore, recovery of them is potentially economically feasible. For this purpose, ceramic membranes have been tested [6, 7]. Sanmartin et al. [8] used a ceramic membrane with a cut-off coefficient of 10 kDa. Separation was connected with one diafiltration, and the resulting retentate was dried and frozen. Using this method, they obtained whey protein concentrate powder (WPC). However, this procedure generates co-products with a high content of lactose that have to be utilized or disposed.

The most common way to bioremediate whey (and similarly rich organic by-products) is through the application of activated sludge. This method is relatively cheap and technically simple. Activated sludge works via a consortium of different microorganisms, mainly bacteria from the genera *Pseudomonas*, *Acinetobacterium*, *Aeromonas*, *Zooglea*, *Enterobacteriaceae*, *Flavobacterium*, *Achromobacter*, *Micrococcus* and *Bacillus*, as well as protozoan ciliates of the genera *Vorticella*, *Paramecium*, *Aspidisca*, and *Suctoria* and flagellates of the genera *Trigonomonas*, *Tetramitus*, and *Bodo* [9]. This microbial diversity results in activated sludge activity that can change at any time, particularly as the load input changes. Usually, activated sludge works more stably at a high dilution [10]. Malaspina et al. [11] reported that 1 g of activated sludge was able to oxidize 0.22 mg of organic components in whey in 5 days, a yield that was not satisfactory. Another disadvantage is the release of microbes into the environment, which can contribute to further growth and cause contamination and environmental risk.

One alternative is to use a monoculture. On the one hand, this strategy requires sterility and may be more expensive. On the other hand, a biodegradation process with one microorganism can facilitate the production of valuable co-products. For example, the metabolism of *Bacillus licheniformis* uses lactose and produces lactic acid [12, 13]. Mehaia et al. [14] used *Kluyveromyces fragilis* to obtain ethanol at the concentration of 30–60 g/dm^3 during the biodegradation of the solution of synthetic lactose (50 g/dm^3). The efficiency of this process was improved by concentrating the microorganism on the membrane. Other authors have also shown the advantages of membrane separations integrated with microbial processes [15, 16].

In the present paper, the biodegradation of sweet whey has been reported, especially compounds contained in the permeate after the ultrafiltration of whey proteins. Experiments were performed in a continuous stirred-tank reactor with a *B. licheniformis* strain. Kinetic studies are the first step in the membrane bioreactor project.

2. MATERIALS AND METHODS

To decrease the natural turbidity of whey (due to residual casein clot and fat), the whey was centrifuged at 3000 g and 4 °C for 20 min (Hettich Zentrifugen Universal 320R, Germany). Then, $CaCl_2$ (CAS: 10043-52-4, POCH, Poland) was added at 2–5 °C, and pH was raised to 7.3 using 6 M NaOH (CAS: 1310-73-2, POCH, Poland). Finally, the whey was heated to 55 °C and held at this temperature for 8 min. The suspension was then cooled and centrifuged at 9000 rpm for 20 min [17]. The bacterial cell content was measured spectrophotometrically (Hitachi, USA) at $\lambda =$ nm using the standard curve:

$$X = 0.389\lambda_{550} \tag{1}$$

where: X – biomass concentration, g/dm^3 and λ – wavelength, nm.

The protein concentration was measured using the Lowry colorimetric method [18] with a standard curve:

$$C_{\text{protein}} = 0.396\lambda_{750} \tag{2}$$

where: C_{protein} – protein concentration, g/dm^3.

The lactose concentration was measured using the DNS method [19] with a standard curve:

$$C_{\text{lactose}} = 2.35\lambda_{550} \tag{3}$$

where: C_{lactose} – lactose concentration, g/dm^3.

Batch process. To develop the growth kinetics of *B. licheniformis* (PCM-1849, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw, Poland), a series of whey solutions with lactose from 0.8 to 8.8 g/dm^3 was prepared. The protein concentration was held proportional to the lactose concentration; the initial ratio was 1:4. The culture was hold at 34 °C and permanently mixed in a water shaker (Elpin plus, Poland). The culture was sampled at regular intervals, and the microorganism content was measured directly before samples for Lowry and DNS analyses were centrifuged at 6000 rpm for 10 min (Hettich Zentrifugen Eba 20, Germany). The samples were measured in two replicates.

Continuous process. The feed tank (Z1) was charged with the solution of whey using sterile distilled water. The lactose content in the dosed stream was between 1.4 and 3.3 g/dm^3 (with a protein content between 0.5 and 1.2 g/dm^3, respectively).

The installation scheme is presented in Fig. 1. The feed was dosed using a gear pump P1 (gear pump, Cole-Parmer Instrument Company, USA) into a thermostatic (37 °C) reactor with a mechanical stirrer (M1). The intensity of mixing was 70 rpm.

Removal of the culture mixture occurred once it reached level h1, and the mixture was sucked with a pump P2 (gear pump, Cole-Parmer Instrument Company, USA). The culture was aerated with a compressor S1 (HL275/50 Specair, Netherlands), and the stream of air was 1.5 dm^3/min. Culture purity was monitored by the daily inoculation of agar plates (N9405, Fluka Analytical, USA).

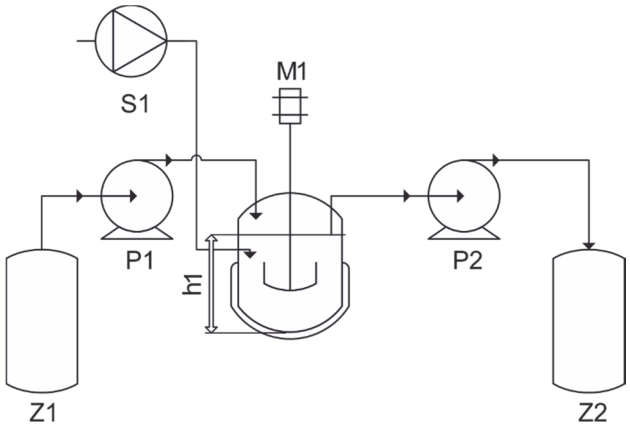

Fig. 1. Installation used for biodegradation of whey: Z1 – whey tank, P1 – dosing pump, S1 – compressor, M1 – mechanical stirrer, P2 – receiving pump, Z2 – tank with culture mixture

The continuous process was conducted with a residence time from 3.7 to 37.1 h. As was established, a steady state emerged after four volume exchanges, which amounted to 2.6 dm^3. After this time, a sample was taken for analysis and centrifuged at 6000 rpm for 10 min (Hettich Zentrifugen Eba 20, Germany) before performing the Lowry and DNS assays. These analyses were performed in duplicate.

Detection of lactic acid. To detect lactic acid, we used a method based on Uffelman's reagent, a 1 M solution of $FeCl_3$ and 2% pure phenol in water. The reagent is violet in colour; when applied to a sample, a yellow colour indicates the presence of lactic acid [20]. The test was performed on culture samples after the biodegradation process.

3. RESULTS AND DISCUSSION

Studies of the kinetic growth of *B. licheniformis* in a batch system showed that a high initial concentration of lactose (above 5.4 g/dm^3) resulted in a long death phase (almost 200 h). The most likely reason for this behaviour is inhibition by one of the compounds in the whey or one of the intermediate products formed during lactose or protein utilization. During the experiments, an increase in cell number and a decrease

in lactose and protein concentrations were observed. Sample plots are shown in Fig. 2. For diluted whey (15–30-fold), the bacterial strain entered the logarithmic growth phase quickly. The stationary phase lasted 4–5 h and did not depend on lactose content. The almost linear decrease in lactose content (and the much slower decrease in peptide content) is concomitant with an increase in biomass concentration. When lactose was exhausted, the culture entered the death phase. However, a slight decrease in the protein content was still observed. Changes in the protein concentration during culturing were so small that it was not possible to determine the growth kinetics relative to this component.

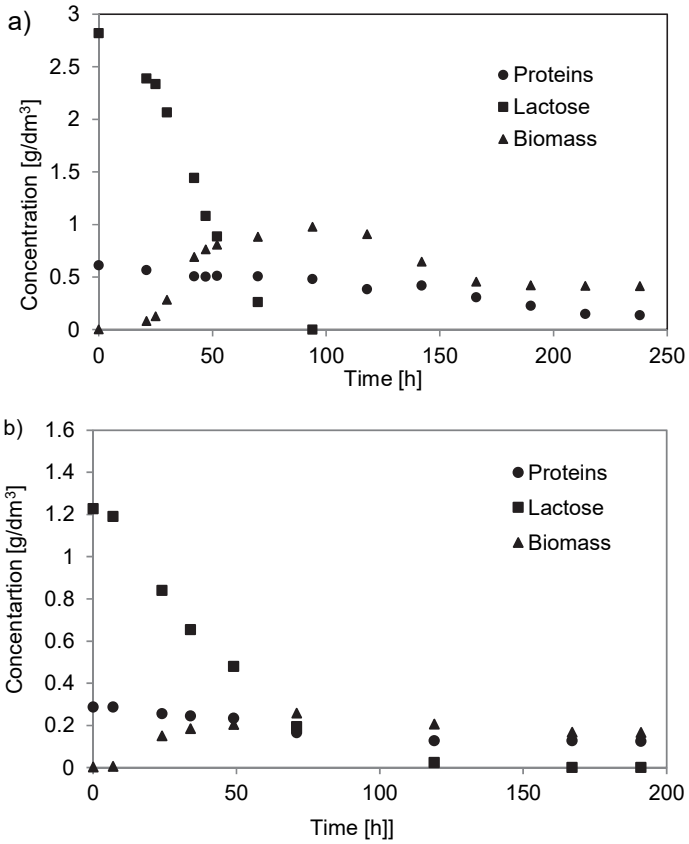

Fig. 2. Lactose, protein and cell concentrations during whey degradation. The initial concentration of lactose 2.82 g/dm^3 (a) and 1.23 g/dm^3 (b), the initial concentration of protein 0.61 g/dm^3 (a) and 0.29 g/dm^3 (b)

As expected, lactose concentrations above 2 g/dm^3 resulted in substrate inhibition. This type of inhibition can be described by the Luong equation [21] with parameters calculated from experimental data (with a relative error of 13.05%)

$$\mu(L) = \frac{\mu_{\max} C_{\text{lactose}}}{K_m C_{\text{lactose}}} \left(1 - \frac{C_{\text{lactose}}}{K_l}\right)^n = \frac{2.29 \times 10^{-4} C_{\text{lactose}}}{4.79 + C_{\text{lactose}}} \left[1 - \frac{C_{\text{lactose}}}{6.03}\right]^{1.1} \tag{4}$$

where: $\mu(L)$ – specific rate of growth (relative to lactose concentration), 1/s, $\mu_{\max}$ – maximum growth rate, 1/s, K_i – constant in Luong's equation, g/dm^3.

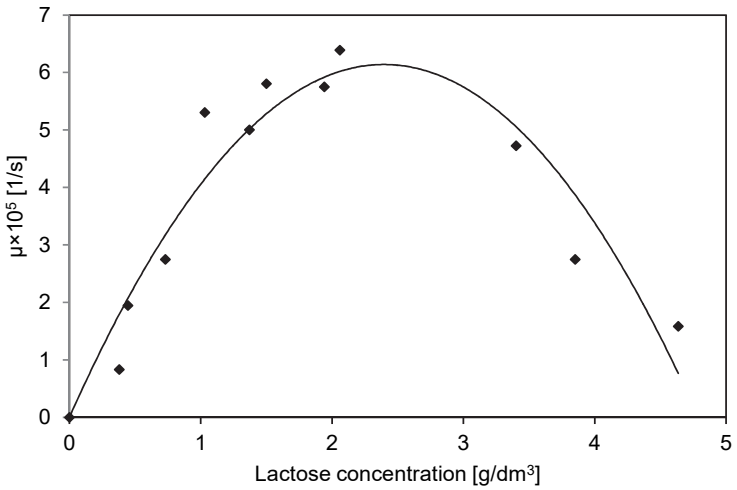

Fig. 3. Kinetics of growth of *B. licheniformis* on whey in dependence of lactose concentration

The investigation of biodegradation in a continuous stirred-tank reactor was based on the research performed in a batch system. These data demonstrated that lactose concentrations exceeding 2 g/dm^3 (and/or protein concentrations of 0.6 g/dm^3) had a negative effect on the growth rate. The observed specific growth rate of *B. licheniformis* reached a maximum of $6.2 \cdot 10^{-5}$ 1/s, and this value was taken into account in the design of the continuous process.

The concentrations of lactose and protein in the input and output streams at residence times from 3.7 to 37.1 h are shown in Fig. 4. Although the metabolism of lactose is dominant, the *B. licheniformis* strain used is able to respire nitrate [22] (Fig. 5). The utilization of organic nitrogen by bacteria is not common, and this feature is extremely valuable in whey biodegradation.

The relationship between lactose concentration and growth rate was comparatively similar to data obtained from batch cultures. In agreement with the accepted theory [23, 24], time points were obtained on the rising side of the plot, and the resulting kinetics were expressed using Monod's equation (Eq. (5)) over a narrow substrate concentration range (at lactose concentrations below 2.25 g/dm^3) (Fig. 6).

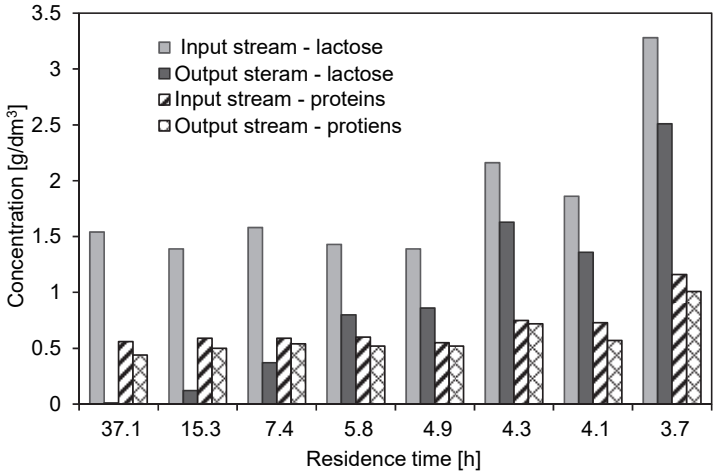

Fig. 4. Concentrations of lactose and protein
in the input and output streams at various residence times

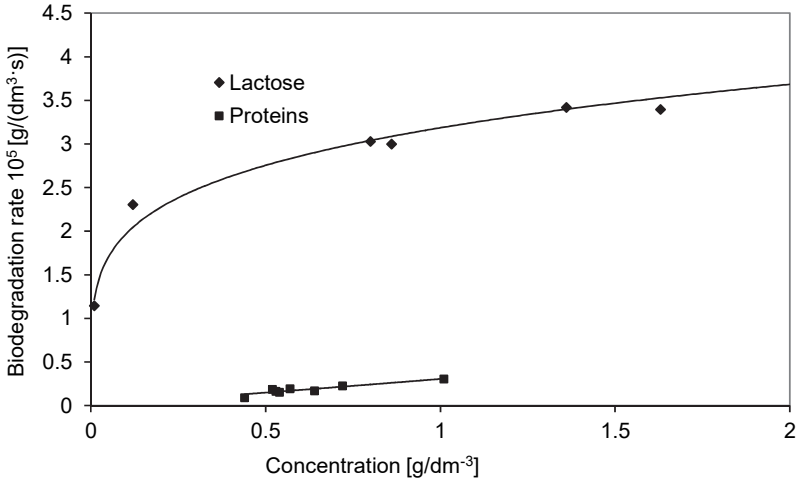

Fig. 5. Rates of biodegradation of lactose and protein with *B. licheniformis*
at an average biomass concentration of approximately 0.2 g/dm^3

The constants were estimated (with a relative error of 4.4%) to be $K_m = 0.45$ g/dm^3 and $\mu_{max} = 8.52 \cdot 10^{-5}$ 1/s.

$$\mu(L) = \frac{\mu_{max} C_{lactose}}{K_m + C_{lactose}} = \frac{8.52 \times 10^{-5} C_{lactose}}{0.45 + C_{lactose}} \tag{5}$$

where: K_m – Monod's constant, g/dm^3.

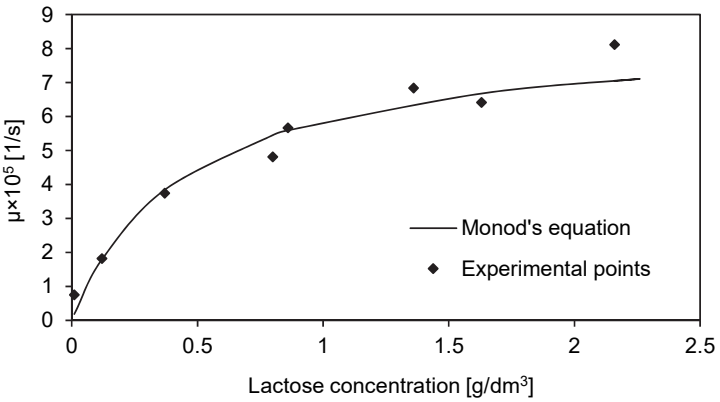

Fig. 6. Monod description of *B. licheniformis* growth on whey

The average value of the biomass yield coefficient [25, 26] relative to lactose consumption Y_{XL_R} as expressed in Eq. (6) was 0.32.

$$Y_{XL\_R} = \frac{X_{\text{reactor}} - X_{\text{input}}}{C_{l\_\text{input}} - c_{l\_\text{reactor}}} \tag{6}$$

where: Y_{XL_R} – biomass yield coefficient (relative to lactose concentration) in CSTR, g/g, X_{reactor} – biomass concentration in the reactor (CSTR), g/dm^3, X_{input} – biomass concentration in the input stream (CSTR), g/dm^3, C_{l_input} – lactose concentration in the input stream of the CSTR, g/dm^3, C_{l_reactor} – lactose concentration in the reactor (CSTR), g/dm^3.

Relative to protein consumption, the average biomass yield coefficient was 2.8 (in the case of multicomponent systems, the biomass yield coefficient relative to one component may be higher than unity).

Finally, the dependence of the lactose conversion rate on lactose concentration was expressed by the following equation:

$$r_L = \frac{X\mu(L)}{Y_{XL\_R}} = \frac{8.52\times10^{-3} C_{l\_\text{reactor}} X}{0.32\left(C_{l\_\text{reactor}} + 0.45\right)} \tag{7}$$

where: r_L – rate of lactose conversion, g/(dm^3·s).

The rate of protein conversion was significantly lower and negligible compared to that of lactose conversion. However, this rate was expressed by the following equation:

$$r_p = \frac{X\mu(P)}{Y_{XP\_R}} = \frac{8.52\times10^{-5} C_{p\_\text{reactor}} X}{2.80\left(C_{p\_\text{reactor}} + 0.45\right)} \tag{8}$$

where: r_P – rate of protein conversion, g/(dm^3·s), Y_{XP_R} – biomass yield coefficient (relative to protein concentration) in CSTR, g/g, $\mu(P)$ – specific rate of growth (relative to protein concentration), 1/s, $C_{P\_reactor}$ – protein concentration in the reactor (CSTR), g/dm^3.

Additionally, the ability of the strain to produce lactic acid was examined at the end of fermentation. The yellow colour in a sample treated with Uffelman's reagent indicated the presence of lactic acid. Whey biodegradation concurrent with lactic acid production will be the aim of our future studies.

4. CONCLUSIONS

Whey is a burdensome by-product produced in large amounts by the dairy industry. Lactose, which is responsible for a high biological load of whey, can be recovered by the use of nanofiltration processes [27, 28]. However, industrial application of this process in the production of food or pharmaceuticals is not sufficient to separate lactose from whole whey.

Additionally, whey is a source of valuable proteins with health-promoting properties [29]. The recovery of these proteins does not significantly decrease the COD coefficient (only approximately by 10 g O_2/dm^3), and co-products from the membrane-based recovery of whey proteins still need to undergo bioremediation.

This co-product stream rich in lactose and macropeptides was successfully biodegraded in a continuous stirred-tank reactor with a *B. licheniformis* strain. The bacteria effectively decreased the lactose content, and with long residence times (above 30 h), we observed an almost total decrease in lactose content as required by the Polish Ministry of Environmental Protection. The COD index in the output stream was 7.6 mg O_2/dm^3, an allowed and safe value. The residence time may be greatly shortened by the application of a membrane bioreactor [30], as planned in our future research.

Beyond desirable environmental protection, this approach may also yield economic benefits because there is the possibility to co-produce lactic acid, which is used widely in the cosmetic and food industries [31, 32].

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