Possibilities of butyric acid production by butanol oxidation with *Gluconobacter oxydans* coupled with extraction.

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In this work the production of butyric acid by oxidation of butanol with *Gluconobacter oxydans* CCM 1783 has been studied. Butanol was added into a bioreactor in portions (0.15 mol·l^{-1} per addition). After 75 hours of biooxidation a concentration of butyrate of 0.56 mol·l^{-1}. representing 93.3 % of theoretical yield, was achieved. The productivity for butyrate was 0.66 g·l^{-1}·h^{-1}. In order to decrease the inhibitory effect of product and to achieve higher butyrate productivity, the possibility of butyric acid extraction with Hostarex A327 (w = 20 %) in oleylalcohol was studied. For higher extraction efficiency, the fermentation broth should be acidified to at least pH 5 before extraction. Sufficient butyrate concentration in the stripping solution could be reached with a higher volume ratio of Hostarex A327 (w = 20 %) in oleylalcohol to stripping phase.

Key words:
Butyric acid, butanol, extraction, *Gluconobacter oxydans*, oxidation, bioconversion

Introduction

Butyric acid and some of its esters are used in the food and perfumes industries because of their aromatic properties.[1–2] This acid is also used in the pharmaceutical industry. Many processes have already been proposed for butyrate production by fermentation during the first half of this century.[3] Nevertheless, fermentation processes have not yet been used commercially because of the low product mass concentration in the fermentation mash (20–30 g·l^{-1}) and because of the acetate which is produced simultaneously with butyrate.[4] Presently, butyric acid is produced mostly from the petrochemical feedstocks.[5] However, the use of butyric acid or its esters as additives, in particular in the food or cosmetic industries, makes its origin important as consumers have preferences for natural products.

Production of acids by bacterial bioconversion of primary aliphatic and aromatic alcohols is the alternative possibility to the common fermentation process. Earlier studies have shown the strong oxidative potential of *Acetobacter* for any kind of alcohol substrate, e.g. primary aliphatic or aromatic alcohols, secondary alcohols, polyalcohols, and cyclic polyalcohols.[6–9] Primary aliphatic saturated monoalcohols, with up to six carbons on the main chain, can be oxidized to their corresponding acids with members of *Acetobacter* and *Gluconobacter* genera.[10] Moreover, *Acetobacter* was used for successful isovaleraldehyde production from 3-methyl-1-butanol (isoamyl alcohol). Other alcohols were transformed into their aldehydes, too.[11] *Acetobacter pasteurianus* was studied for its potential application in the enantioselective oxidation of alcohols.[12] Many authors have demonstrated that strains of *Acetobacter* and *Gluconobacter* are able to transform alcohols into acids. However, studies on optimal conditions for these biotransformations are scarce with the exception of acetic acid, although other organic acids are of great interest to the food, cosmetic and pharmaceutics industries.[10] Recently the production of propionic acid by the biooxidation of propanol with *Gluconobacter oxydans* was also described and partially optimised.[13] Advantages of such acid production compared with common fermentations processes are higher product yields, shortened time for production, higher final product concentrations and negligible by-product formation.[10,13] Nowadays, processes for producing carboxylic acids by oxidation of alcohols or aldehydes, using microorganisms of the genera *Saccharomyces, Hansenula, Pichia, Candida* or *Kluyveromyces*, have also been described.[14]

It has been shown that butyric acid could also be produced by the biotransformation of butanol.[10] In order to obtain a "natural" acid, butanol should have a natural origin (e.g. from acetone-butanol fermentation), too. The important problem, which needs to be solved, is an on-line removal of butyric acid from the fermentation broth because of its inhibitory effect on the bacterial cell. It has been shown that the concentration of undissociated butyric acid of 0.048 mol.l^{-1} is inhibitory to the cell growth and activity.[15] Separation process, which seem to be efficient for biotechnological butyric acid production, includes reactive extraction or pertraction (extraction combined with the stripping of organic phase). These tech-

niques were used by traditional butyrate production with *Clostridium spp.*[16,17]

The fermentative production of butyric acid from sugar has already been described and optimized.[4,16–20] In spite of much effort this process is still inefficient and expensive and cannot compete with petrochemical production of butyric acid. Oxidation of butanol is another possibility of butyrate production. This approach has been only partly studied.[10] One example of successful biotransformation is the oxidation of propanol to propionate. The final concentration of product was doubled and production time was approximately three times shorter than in a traditional anaerobic propionate production in sugar fermentation by *Propionibacterium spp.*[13]

Investigation of the potential of the oxidative formation of butyric acid, as a more efficient method for butyrate production, compared with the traditional production procedure and a possibility of its extraction from fermentation broth, were the aims of this work.

Materials and Methods

Microorganism and cultivation media

The strain used in this study was *Gluconobacter oxydans* CCM 1783 (=ATCC 621). Cells were maintained on a slant agar and transferred monthly. The slant agar consisted of: yeast autolysate (IMUNA, Šarišské Micha¾any, Slovak Republic), 10 g.l^{-1}; glucose, 100 g.l^{-1}; $CaCO_3$, 20 g.l^{-1}; agar (OXOID, Basingstoke, United Kingdom), 25 g.l^{-1}; and distilled water. For the cultivation, the following medium was used: glycerol (PALMA-TUMYS, Bratislava, Slovak Republic), 5 g.l^{-1}; yeast autolysate, 5 g.l^{-1}.

Cultivation procedure

The strain maintained on an agar slant was transferred to the cultivation flask. For both precultures, 0.5 l cultivation flasks filled with 0.1 l of medium, were used. Flasks were incubated on a rotary shaker (180 rpm, 30 °C). The culture inoculated from an agar slant was incubated until it reached the exponential phase (16–24 h). The first preculture in the exponential phase was used for inoculation (φ = 5 %) of the second preculture. Cells in an exponential growth phase after 11–12 h were transferred into the bioreactor. The fed-batch oxidation was performed in a 5 l bioreactor (LF-2, Laboratory Instruments, Praha, Czech Republic). The sterile bioreactor was filled with 2.7 l of sterile medium and 0.3 l of the exponential phase inoculum from the second preculture. Cultivation was performed at 30 °C, with a stirrer speed of 500 rpm, aeration at 3 l·min^{-1}, and a pH of 6.0 (γ = NaOH 15 %). The gas outlet from the bioreactor was passed through a condenser at –2 °C.

Extraction experiments

Extraction measurements were performed in double walled separation funnels with an internal volume of 0.25 l. The funnels were filled with 0.025 l of HOSTAREX A327 (w = 20 %) (MERCK, Germany) in oleylalcohol (HOECHST, Germany) as organic phase and with 0.025 l of aqueous butyric acid (Reachim, Russia) solution with butyrate concentrations in the range from 0.219 mol·l^{-1} to 6.262 mol·l^{-1}, or with 0.025, 0.05, 0.1 and 0.15 l of solution with butyrate concentration 0.219 mol·l^{-1} and 1.044 mol·l^{-1}, respectively. Funnels were then automatically shaken for 5 hours at 30 °C without pH adjustment. The phases were separated after 12 hours of settling at the same temperature. Butyric acid was stripped from the organic phase into the aqueous solution with the addition of a molar excess of NaOH. The concentration of butyrate was determined after acidifying with HCl (pH) by gas chromatography. The content of residual butyrate in the aqueous phase was measured in the same way. The distribution coefficient was then calculated as a ratio of butyrate concentration in organic and aqueous phase, respectively.

Extraction experiments with butanol solution, model butyrate-butanol mixture and cultivation medium (after cell separation, 4000 mg, 10 min) in different stages of biooxidation process respectively, were performed in 0.03 l test-tubes with 0.005 l of organic phase, and 0.005 l of the defined aqueous solution. pH was adjusted to 5.0 by addition of NaOH. Tubes were then automatically shaken at 30 °C for 5 hours. Phases separation and stripping were performed as described above.

Analytical

Biomass concentration was expressed as the optical density of bacterial suspension measured at 650 nm in a 1 cm cell in a Spekol-11, spectrophotometer (Zeiss, Germany). Correlation of biomass concentration and absorbance (A_{650}) was linear in the absorbance range 0.0–0.3. More concentrated samples were diluted with distilled water before measurement.

Butyric acid concentration in samples from bioreactor was determined by means of an electromigrating method using the isotachophoretic analyser ZKI-02 (LABECO, Slovak Republic). Analyses were performed in the capillary 0.8 x 150 mm at a driving current of 250 μA using a conductometric detector. The electrolytes used were: leading 10 x 10^{-3} mol.l^{-1} hydrochloric acid, 22 x 10^{-3} mol·l^{-1} 6-aminocaproic acid, w = 0.1 % mHEC, and terminating 5 x 10^{-3} mol·l^{-1} caproic acid. The concentration of butyrate was calculated according to calibration curve.

The substrate-dependent oxygen consumption rate was determined polarographically with a Clark-type electrode (Chemoprojekt, Satalice, Czech Republic). Measurements were performed in a

thermostated cell, filled with buffer and a suspension of bacterial cells, collected from the bioreactor. This method has been fully described in a previous paper.[13]

Both, butyric acid and butanol in samples from extraction experiments and butanol in samples from biooxidation, were determined by gas chromatography under the following conditions: gas chromatograph CHROM 5 (Laboratory Instruments, Praha, Czech Republic), column 1.5 (length) x 0.003 m (internal radius) packed with PORAPAK Q, carrier gas N_2 at a pressure of 160 kPa, flowrate of H_2 0.026 $l \cdot min^{-1}$ and air 0.5 $l \cdot min^{-1}$, temperature of FID detector and injection 240 °C and column temperature 220 °C. The concentration of butanol was determined after proper dilution according to the calibration curve. Samples for butyrate assay were acidified with HCl (pH). Propionic acid (LACHEMA, Brno, Czech Republic) was used as an internal standard. Butyrate concentration was calculated using the method of an internal standard.

Results and Discussion

Biooxidation of butanol to butyric acid

In this work the fed-batch biooxidation of butanol to butyrate with *Gluconobacter oxydans* CCM 1783, was performed. As the butanol has an inhibitory effect on production and cell respiration, the fed-batch operation mode was chosen. At the beginning no butanol was present in the medium. After depletion of glycerol the dissolved oxygen increased (tenth hour of the process) and at this point butanol to a final concentration 0.15 $mol \cdot l^{-1}$, was added. Butyrate concentration was at first registered in the tenth hour of the process (Fig. 1). When all the butanol was consumed, a steep increase of dissolved oxygen tension was observed and a fresh portion of butanol was added into the bioreactor. The concentration of butyrate increased untill the end of the process. The decrease in butyrate productivity during the biotransformation could be explained by the inhibitory effect of product, which concentration at the end reached 0.56 $mol \cdot l^{-1}$ (49.2 $g \cdot l^{-1}$ of the pure acid). Similar values of the inhibitory butyric acid concentration has been already described for bacteria of the genus *Clostridium*.[15]

The butyrate concentration achieved by our approach was higher (49.2 $g \cdot l^{-1}$) than usually reported in the literature for traditional production of butyrate from sugars (30–40 $g \cdot l^{-1}$)[19,20]. Also the yield of product was 93.3 % of the theoretical value, which cannot be achieved by the fermentation of sugars. These results were repeatable over several experiments (data not shown). On the other hand productivity, calculated here from the first addition of butanol till the end of the process (75 h), was lower (0.66 $g \cdot l^{-1} \cdot h^{-1}$) compared with the commonly used system (0.8 $g \cdot l^{-1} \cdot h^{-1}$)[20] . One possibility, to improve our process is to remove the inhibitory product, which probably leads to a low production rate especially towards the end of the experiment.

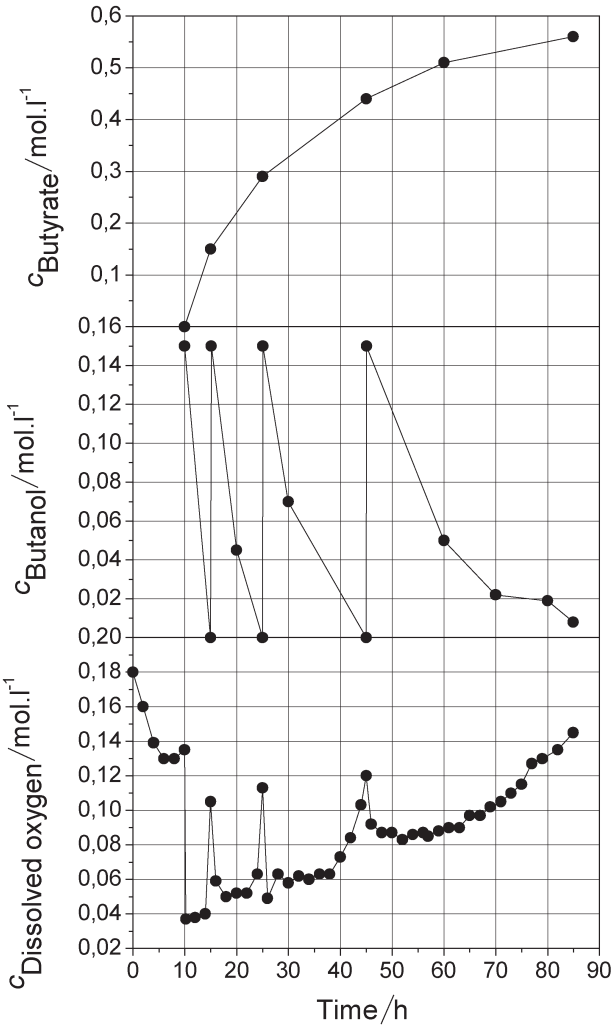

Fig. 1 – *Fed-batch butanol biotransformation to butyric acid using Gluconobacter oxydans CCM 1783 at 30 °C, pH 6.0, stirrer speed 500 min^{-1}, aeration 3 $l \cdot min^{-1}$.*

Extraction experiments

Extraction of butyrate with several solvents has been already described in a few previous works.[16,17,21] Choice of the organic phase for this study was influenced by two factors, biocompatibility and extraction ability. Hostarex A327 (w = 20 %) in oleylalcohol was described in previous works as the organic phase, which is biocompatible with bacteria of the genus *Clostridium* and *Lactobacillus*.[18,22] This organic phase also has a satisfactory extraction efficiency.[18] Therefore, it was chosen for our further experiments.

Dependence of the distribution coefficient on initial concentration of butyrate in the aqueous phase, showed a decreasing trend with increasing concentration. This decreasing tendency has been

shown also in mixtures of Hostarex with other long chain alcohols.[21] For the butyrate concentration, reached at the end of the biooxidation process, of 0.54 mol·l^{-1} the value of this coefficient was approximately 11 (Fig. 2). The increase of butyrate concentration in the organic phase, with increasing initial volume ratio of the aqueous and organic phase, is a concequence of the mass balance under equilibrium conditions. Consequently, butyrate is obtained in the stripping solution at higher concentrations (Fig. 3) and hence, concentrated butyric acid solution can be obtained from the fermentation broth with increase of volume ratio of aqueous and organic phase in the extraction process.

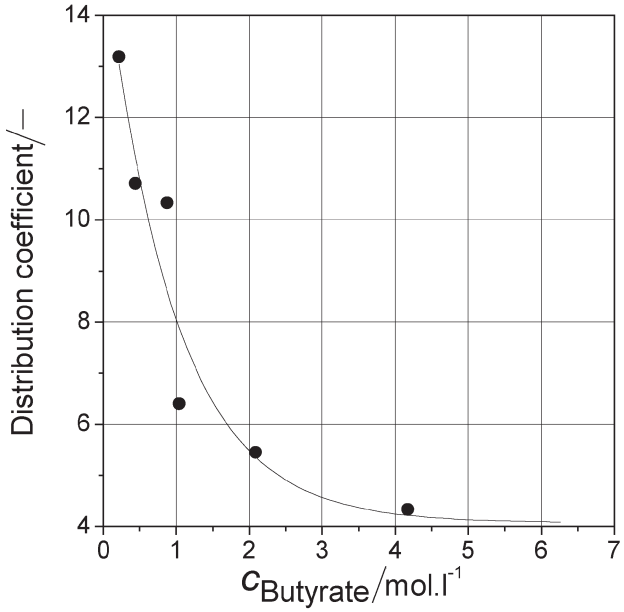

Fig. 2 – *Distribution coefficient of butyrate between water and Hostarex A327 (w = 20 %) in oleylalcohol against initial butyrate concentration in aqueous phase.*

Extraction conditions should be also considered. All previous extraction experiments were performed at pH. Biotransformation runs were performed at pH 6.0. As it has been already published, the distribution coefficient of butyric acid between water and Hostarex A327 (w = 20 %) in oleylalcohol is too low (approximately 1) at this pH value. At the pH 5 this value was higher than 3.[18] That is already enough for satisfactory extraction. Hence, pH 5 was chosen for all further extraction experiments. Therefore, the extraction of butyric acid from the fermentation broth should be performed in an external loop with appropriate pH adjustment or the biotransformation process should run at lower pH.

The next set of experiments was concentrated on the observation of the extraction and pertraction of butanol using Hostarex A327 (w = 20 %) in oleylalcohol, as it is the substrate for biooxidation. The distribution coefficient for butanol showed no dependence on its concentration. Its value was approximately 3. At pH 5 the organic phase extracts butanol and butyrate with the same efficiency. On the other hand, the pertraction (extraction coupled with stripping) experiments with the model mixture of butanol and butyrate and with fermentation broth, showed respectively that the whole amount of butyrate, but only 20 % of butanol would be stripped from the organic phase. These results were similar for the model mixture and for the fermentation broth, respectively (Table 1, 2). The butyrate concentra-

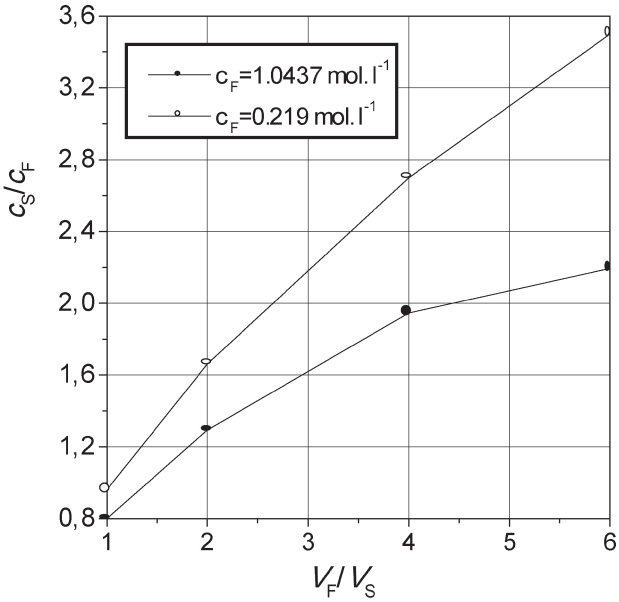

Fig. 3 – *Ratio of butyrate concentration in Hostarex A327 (w = 20 %) in oleylalcohol after 5 h of extraction at 30 °C to initial butyrate concentration in aqueous phase against initial volume ratio of aqueous to organic phase.*

Table 1 – *Butyrate and butanol concentration in the aqueous phase (model mixture) at the beginning of extraction, after 5 h of extraction, and in the stripping solution, NaOH (2 mol·l^{-1}).*

	[a]c_{start} mol·l^{-1}	[a]$c_{equilibrium}$ mol·l^{-1}	$c_{stripping\ solution}$ mol·l^{-1}	[b]Recovery Y/%
Butyrate	0.114	0.023	0.092	101
Butanol	0.054	0.0125	0.0075	40
Butyrate	0.341	0.085	0.250	98
Butanol	0.150	0.0375	0.0225	40

[a] Concentration of butanol and butyrate in model mixture at the beginning of extraction and at equilibrium (after 5 h).
[b] Yield of recovered butyrate and butanol from the organic phase.

Table 2 – *Butyrate and butanol concentration in the aqueous phase (fermentation broth) at the beginning of extraction, after 5 h of extraction, and in the stripping solution, NaOH (2 mol·l^{-1}).*

	[a]c_{start} mol·l^{-1}	[a]$c_{equilibrium}$ mol·l^{-1}	$c_{stripping\ solution}$ mol·l^{-1}	[b]Recovery *Y*/%
Butyrate	0.097	0.021	0.074	98
Butanol	0.000	------	------	------
Butyrate	0.227	0.061	0.165	100
Butanol	0.040	0.011	0.006	43
Butyrate	0.500	0.141	0.356	99
Butanol	0.050	0.014	0.007	42

[a] Concentration of butanol and butyrate in the fermentation broth at the beginning of extraction and at equilibrium (after 5 h).

[b] Yield of recovered butyrate and butanol from organic phase.

tion 0.5 mol·l^{-1} in the fermentation broth dropped after extraction to 0.141 mol·l^{-1}. This concentration has not apparent inhibitory effect to our bacteria strain. Such medium could be returned into the bioreactor after fresh butanol addition for the next biotransformation. After stripping of the organic phase there was founded 0.356 mol·l^{-1} of butyrate in the stripping solution. The coextraction of butanol complicates integration of pertraction and biooxidation. The butanol concentration is low In the stripping but organic phase should be regularly regenerated. This fact makes the whole process more complicated and expensive. On the other hand, there is a possibility to perform the extraction discontinuously exactly in the time of entire depletion of butanol, prior the next substrate addition. Hence, the inhibitory effect of butyrate will be, at least partially, avoided and the productivity will be expected to be more stable. Also the negative effect of butanol cannot be completely removed, the discontinual butanol feed with proper concentration could keep the amount of butanol in the bioreactor under the inhibitory level.

Conclusions

As the common anaerobic fermentative process for butyrate production, using bacteria of genus *Clostridium* and using sugar as a carbon source, is too complicated and not sufficiently effective, the new oxidative approach for butyrate production was studied. Bacteria strains of the genus *Gluconobacter* or *Acetobacter* are usually used for oxidation of different alcohols to their aldehydes or acids.[10,13] The oxidation process is more efficient, for example, for the propionic acid production compared with a common anaerobic fermentation.[13] During the fed-batch biotransformation of butanol using *Gluconobacter oxydans* CCM 1783, the butyrate concentration of 0.56 mol·l^{-1} was reached, and 93.3 % of the theoretical product yield, was achieved. As the high concentration of butyrate towards the end of the biooxidation has an inhibitory effect to the cell activity, the separation of butyrate from medium during oxidation should be considered. The extraction experiments showed that the integration of these two processes is complicated but not impossible. The fermentation broth should be acidified before extraction, or pH value during the oxidation process should be lower than usual, in order to keep extraction efficiency of butyrate sufficiently high. As butanol is coextracted, discontinuous butyrate extraction in an external loop, in the time of the entire butanol depletion, should be considered. High efficiency of butyrate stripping from organic phase using a molar excess of NaOH, promises to obtain high concentrated butyrate solution from the fermentation broth.

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