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REVIEW

Advances in biotechnological production of butyric acid

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The review is focused on several aspects of butyric acid production: butyric acid-producing bacterial strains, the characteristics of the genus *Clostridium* (the bacterium most used for butyrate production), and alternative methods of obtaining butyric acid by alcohol biotransformation. Further, the main metabolic pathways of butyrate production, and possibilities for their control are outlined. Batch, fed-batch or continuous fermentation combined with cell recycle or immobilization are applicable for anaerobic fermentations using *Clostridium* as the production strain. The best process comprises a combination of high cell concentration and slowly growing biomass, in addition to high production selectivity and low inhibitory effects of the end-product. Inhibitory effects may be avoided by on-line removal of the end-product. Extraction alone or extraction combined with simultaneous stripping of the organic phase (liquid membrane) into the second aqueous phase (pertraction) seem to be the most suitable methods for on-line butyrate removal. The biocompatibility and the distribution coefficient of the organic phase under fermentation conditions should be considered before designing a fermentation apparatus. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 153–160.

Keywords: butyric acid; fermentation; oxidation; *Clostridium*; extraction; biocompatibility

Introduction

Butyric acid has several potential applications in industry. Its use in fuel production was originally mentioned in 1923 [46]. Nowadays, its applications in the foodstuffs and beverage industries are widespread. It may be used as the pure acid in the dairy industry, or in the form of esters as a food additive to increase fruit fragrance [4,69]. Other important uses are in the chemical and pharmaceutical industries. Butyric acid and its derivatives (in mixtures with other compounds eg cellulose, acetic acid) play an important role in the plastic materials and textile fibres industries [62].

Butyric acid can be prepared by oxidation of butyraldehyde which has been obtained from propylene by oxosynthesis [63]. However, such a product cannot be considered a substance of natural origin. Another method of preparation is by the extraction of butyric acid from butter. Its concentration in butter ranges from 2% to 4%. It is clear that this kind of procedure is a difficult and an expensive one [70] and cannot compete with the chemical alternative.

In spite of this, butyric acid production from natural sources is very often required. Consumers prefer foodstuff additives or pharmaceutical products containing ingredients of natural origin. They are considered 'healthier' and the customer is ready to pay more for such natural products.

One of the well known alternative methods, applicable for production of butyric acid of natural origin, is fermentation technology. A successful approach should consider several parameters. The first is the right choice of an appropriate microorganism and knowledge of its metabolic pathways. The fermentation process should then be optimized for the needs of the selected microorganism. Downstream processing is also very important. One of the methods of biotechnological production of butyric acid is to join the fermentation process with a simultaneous isolation procedure.

All these parameters have been investigated and described in several publications. This review summarises progress in this field.

Butyric acid as an end product of some bacterial strains

Production strains

There are several bacterial strains which produce butyric acid. Production is mostly an anaerobic process and the producers are strict anaerobes. Production strains belong to the genera *Clostridium*, *Butyrivibrio*, *Butyribacterium*, *Sarcina*, *Eubacterium*, *Fusobacterium* and *Megasphera*. The species *Bacteroides melaninogenicus*, *Treponema phagedenis* and *Peptococcus asacelarolyticus* are also known as butyrate producers [62,74]. The genera *Clostridium*, *Butyrivibrio* and *Butyribacterium* are the mostly used microorganisms (Table 1).

Regarding commercial use, strains of *Clostridium* sp are preferred for butyric acid or butanol production (Table 1). Their productivities are high and relatively stable. They are Gram positive, chemoorganotrophic, strict anaerobes and spore-formers. Strains can be isolated from soil, waste water, animal digestive systems and contaminated dairy products. There are several storage possibilities. Long-term

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Table 1 Examples of butyrate-producing strains and culture conditions

Organism	Carbon source	Culture design
Clostridium butyricum	Glycerol	Batch and fed-batch [61]
	Celulose and wheat straw	Chemostat and batch [12]
	Whey lactose	Batch [86]
	Saccharose	Batch [85]
	Starch	Batch [1]
	D-xylose	Chemostat [38]
Clostridium beijerinckii	Cheese whey lactose	Batch [1]
	Cheese whey lactose	Mixed culture with *Bacillus cereus* [80]
Clostridium pasteurianum	Glucose	Continuous with cell recycle [30]
Clostridium barkeri	Glucose	Batch and fed-batch [33]
Clostridium acetobutylicum	Glucose/glycerol	Chemostat [25]
Clostridium thermobutyricum	Glucose	Batch [91]
Clostridium thermopalmarium	Glucose	Batch [75]
Butyribacterium methylotrophicum	Glucose, lactate, or pyruvate	Batch [71]
	Carbon monoxide	Continual gas sparging [92]
Pseudobutyrivibrio ruminis	Glucose	Batch [28]

storage is possible after lyophilization. Short-term storage is possible in a medium with a minimal content of fermentable sugar, in the form of a spore suspension in sterile water at 4°C [88] or in sterile glycerol solution (25% v/v) at –70°C [8].

Optimal cultivation conditions are 35–37°C, an atmosphere of pure CO_2, N_2 or a 1:9 mixture of N_2 and CO_2 [3] and a pH range of 4.5–7.0. The pH value depends on the objective of the bioprocess, because the pH optima for acidogenesis and solventogenesis differ [27]. *Clostridium* bacteria are able to utilise a wide range of sugars: hexoses, several pentoses and oligo- and polysaccharides. Glucose is the common carbon source for butyrate or butanol production with *Clostridium*, but lactose from whey [1,17,86], saccharose from molasses [85], starch [66,79], potato wastes [26], wheat flour [21], cellulose [12] or dextrose [70] are applicable also (Table 1).

Metabolic pathways and their regulation

Clostridium metabolic pathways of glucose fermentation produce several products. The main products are butyrate and butanol, by-products are acetate and acetone; lactate and ethanol can also be produced in small amounts (Figure 1) [24]. Production of propionate, and 1,3-propanediol from glycerol [2,37,61,96], overproduction of amylolytic [79], and proteolytic enzymes [16] were also detected.

Approximately the same conditions are required for the production of butyrate and acetate. During acetate production, 4 mols of ATP are formed. During butyrate production only 3 mols of ATP are formed. At high growth rates, cells have a higher energetic demand, and they need more ATP. In this case acetate is produced. Butyrate is produced in slower growing cultures. Lactate production is detected under very slow growth conditions [48]. Butyrate production is higher in fed-batch, glucose-limited and slow-growing cultures than in classic batch culture. It is impossible to obtain butyrate as the only product of the bioprocess. The selectivity of the butyrate production can be improved using cell-recycle culture [48,49]. Moreover, acidogenesis is also stimulated by nitrogen and phosphorus limitation [40].

The decisive factors required for the shift from acid to butanol and acetone production include pH, the growth phase of culture and amount of undissociated acids in the fermentation broth [45]. The highest solvent production rates are reached at $pH<5$ and in the stationary growth phase. Fermentation process without pH control can be divided into two phases: acidogenesis until the pH falls to 5 and solventogenesis at $pH<5$ [52]. Recent publications describe a mutant strain of *Clostridium acetobutylicum* which utilizes butyrate, and thus improves solvent production [77,78]. Another possibility for improving solvent production is the use of a low-acid-producing *Clostridium* strain [57].

A shift from acidogenesis to solventogenesis is undesirable during butyrate production. The reasons for such a shift should be known before attempts can be made to avoid it. Activities of enzymes involved in the pathway from acetyl-CoA to butyryl-CoA are important for both butyrate and butanol production (Figure 1) [7,24]. Butyrate will only be produced subsequently if there are sufficiently high levels of the enzymes involved in the pathway from butyryl-CoA to butyrate present [31]. These enzymes are influenced by the ATP concentration and the NADH:NAD ratio. A minimal intracellular ATP concentration and a high NADH:NAD ratio stimulate solventogenesis [26].

Based on the above information, one can make certain conclusions about the optimal conditions of butyrate fermentation. The key enzymes may be influenced indirectly with the change of oxidative and reductive relations in cells or directly by inhibitory compounds. Oxidative and reductive relations may be influenced with methyl viologen [58–60]. The use of rifampicin and chloramphenicol directly affects enzymes in the solvent-producing pathways [89]. Iron limitation is another method of influencing enzyme activity [47].

Butyric acid fermentation

Fermentation approaches

Batch-, fed-batch-, continuous and cell-recycle-fermentations are most frequently used for butyrate production. Results of such experiments offer a deeper insight into strain physiology and behaviour.

Slow cell growth, which may be evoked by carbon limitation in continuous or fed-batch processes, has a positive effect on butyrate productivity and selectivity. Higher butyrate concentrations may be obtained in fed-batch cultures than in continuous cultures. On the other hand, higher productivity may be achieved by use of continuous cultures [49,50]. The continuous process starts with a batch phase, in which the initial concentration of carbon source is utilised. Subsequently, the batch process is switched to the continuous state [23]. An advantage of the continuous culture compared to the fed-batch system is also a possibility

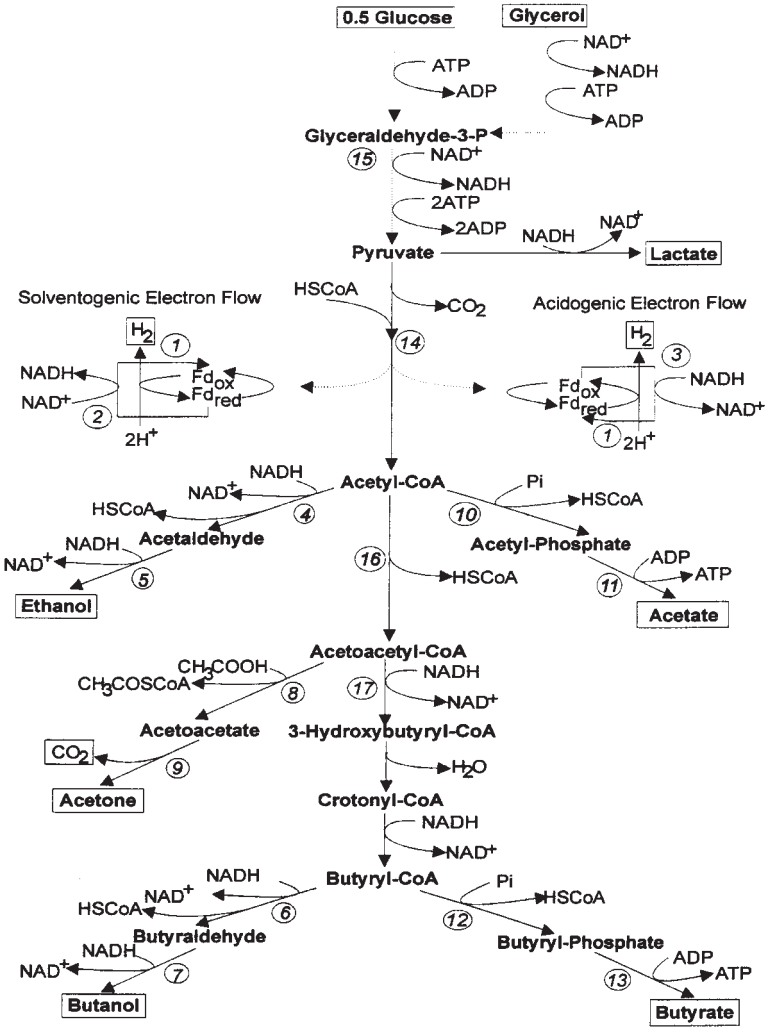

Figure 1 Metabolic pathways of *Clostridium acetobutylicum*: 1, hydrogenase; 2, ferredoxin-NAD reductase; 3, NADH-ferredoxin reductase; 4, acetaldehyde dehydrogenase; 5, ethanol dehydrogenase; 6, butyraldehyde dehydrogenase; 7, butanol dehydrogenase; 8, CoA-transferase; 9, acetoacetate decarboxylase; 10, phosphotransacetylase; 11, acetate kinase; 12, phosphotransbutyrylase; 13, butyrate kinase; 14, pyruvate ferredoxin oxidoreductase; 15, glyceraldehyde-3-phosphate dehydrogenase; 16, thiolase; 17, 3-hydroxybutyrylCoA dehydrogenase [24].

to perform the process during a longer time period. The cell recycle system, together with continuous process-design, is another method for obtaining a slow growing culture of *Clostridium* bacteria with a high production rate of butyrate [48].

In order to achieve high and stable butyrate formation during a continuous fermentation process, at least one parameter should be controlled and maintained. A simple method for estimating the culture state is to measure gas formation. Fed-batch butyrate production with *Clostridium*, controlled by the gas formation rate was more efficient in comparison with conventional constant feeding [20].

Cell immobilization, using polyvinyl alcohol and boric acid, can be applied effectively to the anaerobic process with butyrate productivities higher than in the common continuous process [29,39]. An increase in butyric acid production rate can be achieved during fermentation using immobilised cells by pulsewise addition of different vitamins. The addition of biotin alone may be responsible for a 50% increase in production rate [65].

An alternative approach to butyric acid production is a two-stage fermentation process consisting in the conversion of a lactate salt to salt of another acid (acetic, propionic or butyric acid) by a selected bacterial strain (*Clostridium thermoaceticum*, *Propionibacterium freudenreichii*, *Propionibacterium acidipropionici* or *Butyribacterium methylothropicum* res), the addition of fermentable carbohydrate to the fermentation mixture and its transformation by *Lactobacillus bulgaricus*, *L. delbrueckii* or *L. acidophilus* to lactate salt, followed by conversion of the salt of selected acid into free acid and separation of the free acid from the mixture. The fermentation broth containing the lactate salt is recycled into the first step [11] so that the lactate salt can be transformed again.

Biotransformations of alcohols are a current trend in organic acid production. One of the first attempts to produce butyric acid this way was the oxidation of butanol using bacterial strains of *Gluconobacter* or *Acetobacter*. These strains were also used for propionic acid production from *n*-propanol [81,82]. *Acetobacter aceti* was later

exploited for oxidations of several primary alcohols into organic acids [14]. Several yeast strains eg *Saccharomyces*, *Hansenula*, *Pichia*, *Candida* or *Kluyveromyces* spp have a great potential in the field of alcohol (aldehyde) oxidation to carboxylic acids [90].

The conventional anaerobic production of butyrate faces several problems. Trying to find solutions to three of them has been the subject of many publications. They are: (i) down-stream processing; (ii) simultaneous ester production [21]; and (iii) the inhibitory effect of end-products [5]. Therefore, the fourth section of this review is focused on down-stream processing with emphasis on the extraction and pertraction (extraction with simultaneous stripping) of carboxylic acids.

Inhibition of fermentation by substrates and end-products

Many metabolic pathways are inhibited by their end-products. In the case of *Clostridium*, butyrate, lactate, acetate, butanol, ethanol and acetone have received attention. Undissociated butyric acid passes through the bacterial membrane and dissociates inside the cell. It influences the transmembrane pH gradient and decreases the amount of energy available for biomass growth [34]. Butanol has a negative effect on the fluidity of membranes [6], on membrane function and on ATP levels [91]. Butyrate fermentations are often inhibited by substrate and pH. Excess carbon source is often a reason for osmotic dehydration and pH affects the formation of acids, their form (dissociated, undissociated), membrane transport and cell lysis [15].

Several models for the inhibitory effects of products and substrates have been developed. They use the same basis but differ in details. Here are three examples of such models:

$$\mu = \frac{\mu_{max} * S}{K_s + S + S^2/K_i} * \frac{K_p}{K_p + P} \quad (1)$$

$$\mu = \mu_{max} * \frac{S}{K_s + S + S^2/K_i} \quad (2)$$

$$* \{[1 + (HAc/K_a)^{\alpha}] * [1 + (HBu/K_b)^{\beta}]\}^{-1}$$

$$\mu = \mu_{max} * \frac{S}{K_s + S} \quad (3)$$

$$* [\Pi(1 - C_{Pi}/C_{Pi}^*)] * \frac{1}{1 + (H^+/K_H) + (K_{OH}/H^+)}$$

Where μ is the growth rate; μ_{max} the maximum growth rate; S and P stand for concentrations of substrate and product respectively; K_s substrate saturation constant; K_i and K_p inhibition constants of substrate and product respectively; Hac and HBu, concentrations of acetic and butyric acids respectively; K_a and K_b, dissociation constants of acetic and butyric acids respectively; α, β, K_h, K_{oh} are constants, H^+ is the concentration of H^+, and C_{pi} and C_{pi}* the concentrations of inhibitory product and critical concentration of product, which stops growth of microorganisms completely. The first model includes the inhibition caused by substrate (Haldane equation) and product. The effect is expressed by means of the saturation constant K_p [35]. The influence of the product in the second equation is expressed by means of the acid dissociation constant. This model includes the influence of both acetic and butyric acids [93]. The third model is the most complex one. It takes into account the effects of substrate, all possible products and pH [96].

The equations show the complexity of inhibitory effects. However, some simple conclusions can be made, based on these models and measurements. The inhibitory effect of dissociated acids at levels that can be reached in the process is negligible. The critical inhibitory concentration of undissociated butyrate is approximately 50 mmol L^{-1} [36]. Inhibitory effects of the other by-products eg acetate, butanol, ethanol and acetone appear in a concentration range that is above the concentrations usually reached during the fermentation [91]. The addition of acids has a lower toxic effect than those which are produced by the cells. This phenomenon was explained by the fact that acid concentrations, within acid-producing cells, are higher than when the acids are added externally [96].

It is important to keep the butyrate concentration under the inhibitory level during its production. This condition can be established at high dilution rates during continuous fermentation. However, this approach is not a convenient one because of high product dilution. For down-stream processing, it is important to achieve a butyrate concentration as high as possible. Approaches to achieving this form the subject of the next section.

Fermentation coupled with product isolation

There are several product isolation techniques, which can be combined with fermentation processes. Many published approaches deal with the possibilities of *in situ* or on-line removal of product. These methods have several advantages. They separate the product from cells and the fermentation broth just after its production. The losses of product, usually caused by its interactions with cells and with components of medium, are reduced in this way. Another advantage is the elimination of several separation steps after the fermentation process [22].

Distillation and pervaporation processes are used for isolation of volatile products. They are often inter-connected with acetone-butanol fermentation [51,64]. Application of micro- or ultra-filtration for biomass separation during the fermentation process and subsequent permeate electrodialysis is often applied to the separation of easily dissociable acids (lactic [9,72], acetic or propionic [10] acid or amino acids [44]). Adsorption methods are sometimes used [54]. Extraction is a widely used separation technique. The method is often used also for butyrate separation.

Biocompatibility of solvents and their extractive ability

Several options have to be considered before the butyrate fermentation is coupled with extraction or pertraction. The effect of the extraction step on microorganisms must be considered.

Physical, reactive and supercritical extraction models have been described [68]. The first two methods have been used in connection with the production of organic acid.

Table 2 Commonly used organic solvents and their log P values (defined as the logarithm of a solvent's partition coefficient in a standard octanol:-water mixture) [40], and carriers with examples of their toxicity to some microorganisms

Solvent	Log P	Carrier	Toxicity[a]
Benzene	2.0	Alamine 304	+–
Heptanol	2.4	Alamine 308	+
Toluene	2.8	Alamine 336	+
Styrene	2.9	Adogen 283-D	+
p-Xylene	3.1	Amberlite LA-2	+
Ethylbenzene	3.3	20% TOPO and kerosene	–
Cyclohexane	3.4	20% Hostarex A327 and	–[b]
o-Dichlorobenzene	3.6	oleylalcohol	
Propylbenzene	3.8	Trihexylphosphate	–[b]
Hexane	3.9		
Diphenylether	4.2		
Cyclooctane	4.5		
Isooctane	4.8		
Octane	4.9		
Hexylether	5.1		
Nonane	5.5		
Decane	6.0		
Dodecane	7.0		

[a]Toxicity of carrier in fermentation system with *Propionibacteriun acidipropionici* [76].
[b]Toxicity was determined in a system with *Clostridium butyricum* [87].

Physical extraction is a simple method, in which the organic phase must be regenerated after saturation with the product. The method can be used when the organic phase is used for the next steps, eg esterification of acid with lipase [21,53]. Reactive extraction has higher efficiency, because the organic phase also contains a reactant or carrier. It means that the acid is extracted into an organic phase by physical transport and complexation with the carrier [68]. Several chemicals are commonly used as the organic phase. Authors have considered the biocompatibility of the organic phase (Table 2) [40,76] together with its extractive ability (Table 3) [97].

Biocompatibility of a solvent may be assessed by its log P value. Log P is defined as the logarithm of a solvent's partition coefficient in a standard octanol : water mixture. The higher the log P value, the lower the toxicity [40]. The biocompatibility of the carrier should be determined for each microbial system. The toxicity of the organic phase can be decreased by cell immobilization or by addition of protective substances (eg soybean oil) [94].

Table 3 Distribution coefficient (D) of butyric acid for several organic phases [97].

Organic phase	D
Hostarex A327 (20 wt%) in isodecanol	7.90
Hostarex A327 (20 wt%) in isotridecanol	6.57
Hostarex A327 (20 wt%) in oleylalcohol	6.40
n-Octanol	6.31
Isodecanol	5.60
Isotridecanol	4.82
Di-*n*-butylether	2.96
Oleylalcohol	2.85
Toluene	2.40
Rape seed oil	1.02
Sunflower oil	0.99
n-Alkanes	0.71

Distribution coefficients have already been measured for a substantial group of organic acids [43,83,84]. Coefficients for butyric acid were measured in several solvents [97] (Table 3). The effectiveness of the extraction process and the distribution coefficient depend strongly on pH [32]. The design of an extractive butyrate fermentation should take into account that organic solvents extract only undissociated acids. This means that the distribution coefficient decreases with increasing pH. On the other hand, low pH values cause solventogenesis.

This problem can be partially solved by reactive extraction or by pertraction (membrane extraction). Product is extracted from the fermentation broth and simultaneously stripped from the organic phase into the stripping solution. The organic phase (the so-called membrane) is simultaneously regenerated in this process. The most effective membranes are emulsion liquid membranes, supported liquid membranes, hybrid-liquid membranes and hollow fibre modules [19]. Fermentations coupled with extraction are called two-phase fermentations. Processes coupled with pertraction are called three-phase or membrane fermentations.

One approach for selective separation of dilute products from simulated clostridial fermentation broth is the application of cyclodextrins [73]. There are several types of cyclodextrins, which are able to extract just one substance selectively from a mixture. Because of this special feature, the system can be applied to butyrate or butanol fermentation.

Two- and three-phase fermentations

There are several possibilities for the design of a fermentation aparatus for acid production. The design for butyrate production depends strongly on the bacterial strain used and its ability to grow and produce acids in the presence of an organic solvent. Another crucial point is the pH of the medium. For acidogenesis a pH value higher than 5.2 is required [87]. However, the effect of extraction decreases with increasing pH. This obstacle can be eliminated by a membrane process or by an external extraction loop. There are not many publications dealing with application of extraction or pertraction in butyrate fermentation processes, even though the potential of the method is obvious.

The productivity of lactate was improved from 7 g $(Lh)^{-1}$ to 12 g $(Lh)^{-1}$, during lactate fermentation by using immobilized cells and continuous on-line extraction of lactate in an external loop with Alamine 336 (15% w/w) in oleylalcohol as the organic phase. [95] Improvement of lactic acid fermentation performance can also be increased by a multistage extractive fermentation [42] with cell recycle. An extractive fermentation process, using a hollow fibre extractor with amine-based extractant, was also developed to reduce end product inhibition and to increase the productivity of acetate [67]. This design has also been applied to propionic acid production [41].

Productivity of solvents, in acetone-butanol fermentations with *Clostridium acetobutylicum*, was doubled by the application of microfiltration for separation of biomass

and a pertraction system with supported liquid membrane containing a mixture of oleylalcohol and dodecane as the organic phase for removal of the butyric acid formed [26]. Another interesting and effective design for butanol production is a fluidised bed bioreactor with cells immobilised in χ-carrageenan. Oleylalcohol serves as the extractant which enters the bottom of the bioreactor and which is collected from the top of bioreactor and regenerated [13].

There are few examples of butyrate production in an extractive or pertractive fermentation. In fact, designs described above are also convenient for butyric acid recovery. Extraction of butyrate with oleylalcohol, during a batch fermentation, prolongs the production phase compared to a simple batch process [18]. Use of a pertractive system, with a supported liquid membrane (trioctylphosphineoxid (20% w/w) in *n*-alkanes in a polytetrafluorethylene membrane) for butyrate production, produced a five-fold increase in acidogenesis [55,56].

Further studies have investigated the possibilities of butyrate extraction off-line [21,53]. Off-line extraction has no effect on the fermentation process and its use is less advantageous than on-line extraction. On-line and *in situ* isolation of end product improves the whole production, both the down-stream processing and the fermentation process itself. It concerns mainly end products, such as butyric acids which have inhibitory effects on cell growth and productivity. Extraction is a simple process with wide flexibility and applicability. Because of these characteristics, extraction has great potential in butyrate production as well as in the biotechnological industry in general.

Conclusions

Butyric acid can be extracted from butter but this method is too expensive. It is possible to obtain this acid from a fermentation, as it is the product of the butyrate metabolic pathway of strains of the genera *Clostridium*, *Butyrivibrio*, *Butyribacterium*, *Sarcina* and others. The preferred strain is in the genus *Clostridium*. This strain has two parallel metabolic pathways. Products of the first pathway are acids (butyrate and acetate). This pathway is entitled 'acidogenesis' and products of the second pathway (solventogenesis) are solvents (butanol and acetone). Because a decreased pH and an increase in the NADH/NAD ratio cause the shift from acidogenesis to solventogenesis, the conditions for acid production should be correct. Biotransformation of alcohols into acids with yeast or some bacteria (*Gluconobacter*, *Acetobacter*) is another method for butyric acid production.

A serious problem in the biotechnological production of butyrate is end-product inhibition. Butyric acid has a negative effect on transmembrane pH gradient, and butanol affects membrane fluidity. This problem could not be solved by common fermentation designs. Inhibition effects could be suppressed by on-line or *in situ* product removal. For volatile products such as solvents, distillation and pervaporation are usually used. Electrodialysis may be applied for easily dissociating products, such as amino acids, lactate or acetate.

Extraction and pertraction are most suitable for on-line and *in situ* removal of butyric acid and choice of the organic phase is important. It should be a biocompatible and effective extractant. Addition of reactant or carrier to the organic phase, which should also be biocompatible, increases the distribution coefficient. Such organic phases are, for example, mixtures of Alamine 336 or Hostarex 327 with oleylalcohol. Pertraction processes with a liquid membrane are even better than simple extraction. Liquid membrane (the organic phase) is simultaneously regenerated with aqueous stripping solution, where the product may be concentrated. Fermentation processes combined with pertraction are performed in three liquid phases. The first phase is the fermentation broth, the second is the organic phase and the third is the aqueous stripping solution. These combined processes can be successfully applied to a wide group of biological products including butyric acid.

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