Review

Microbial production of organic acids: expanding the markets

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Microbial production of organic acids is a promising approach for obtaining building-block chemicals from renewable carbon sources. Although some acids have been produced for some time and in-depth knowledge of these microbial production processes has been gained, further microbial production processes seem to be feasible, but large-scale production has not yet been possible. Citric, lactic and succinic acid production exemplify three processes in different stages of industrial development. Although the questions being addressed by current research on these processes are diverging, a comparison is helpful for understanding microbial organic acid production in general. In this article, through analysis of the current advances in production of these acids, we present guidelines for future developments in this fast-moving field.

Introduction: organic acids as building-block chemicals

A major step for the development of a sustainable, industrial society will be the shift from our dependence on petroleum to the use of renewable resources. Bio-refineries enable production of bio-fuels as well as building-block chemicals from biomass as outlined in Box 1. The market share of biotechnological processes for the production of various chemical products is expected to rise from the current level of 5% to 20% by 2010 [1]. In the long run, the use of petroleum could be eliminated and greenhouse gas emissions reduced. These processes are also favorable from a chemical and economic point of view. Functional groups that must be introduced by costly oxidative process steps into naphta are already present in plant materials such as carbohydrates. Organic acids constitute a key group among the building-block chemicals that can be produced by microbial processes (Table 1). Most of them are natural products of microorganisms, or at least natural intermediates in major metabolic pathways. Because of their functional groups, organic acids are extremely useful as starting materials for the chemical industry as outlined for succinic acid in Box 2. It is noteworthy that for many organic acids the actual market is small, but an economical production process will create new markets by providing new opportunities for the chemical industry. For example, succinic, fumaric and malic acid could replace the petroleum-derived commodity chemical maleic anhydride. The market for maleic anhydride is huge, whereas the current market for the organic acids mentioned is small owing to price limitations. Once a competitive microbial production process for one of these acids is established the market for that acid will increase [2].

Consequently, the field that investigates microbial organic acid production is currently moving fast. Here, we summarize the recent developments exemplified by three products: citric acid, which has been on the market for some time, lactic acid, which came to market in large-scale only recently, and succinic acid, which (despite the fact that a feasible industrial bio-process has not yet been developed) has huge potential as a building-block chemical (see Table 2 for an overview of data from scientific literature).

Trends in the field are seen (see below) but, because of the sensitivity of the data between industrial competitors, few process details are known, which impedes sound techno-economic evaluations. Consequently, this review is based on the available scientific literature, which does not entirely reflect the needs of the industry or the current status of ongoing studies. Nevertheless, we set the current trends in academia in context with industrial needs as far as information is available.

Microbial organic acid production: three acids as examples

From Italian lemons to filamentous fungi: citric acid

The oldest microbial process for production of a high-volume, low cost organic acid is the production of citric acid by the filamentous fungus *Aspergillus niger*. Currently the yearly production of citric acid is approximately 1.6 million tons (t) [3]. Unlike most of the other bio-derived acids that are considered industrial products, citric acid was produced industrially before the development of a microbial process. The industrial production relied on extraction from Italian lemons until it was discovered that *Aspergilli* accumulate this acid in high amounts under certain conditions (reviewed by Papagianni [4]). The crucial parameters resulting in efficient production of citric acid by *A. niger* have been determined empirically and include high substrate concentration, low and finite content of nitrogen and certain trace metals, thorough maintenance of high dissolved oxygen, and low pH. The exact definition of these parameters enabled the development of highly efficient biotechnological processes. However, many of the biochemical and physiological mechanisms underlying

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Box 1. Biorefinery

A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power and chemicals from biomass. The biorefinery concept is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum (The National Renewable Energy Laboratory; http://www.nrel.gov/biomass/biorefinery.html).

Currently, ~5% of the total petroleum output from a conventional refinery goes into chemical products; the rest is used for transportation fuels and energy. This ratio is not expected to change in the future [55].

Figure I depicts the concept of biorefineries. In a biorefinery, biomass is defined as any organic matter that is available on a renewable basis and this includes energy crops, agricultural and forestry residues, animal wastes and other organic residues. Different technologies are used for refining depending on the raw materials and the processes for obtaining the desired products [56]. Lignocellulosic feedstocks, for example, can be separated into cellulose, hemicellulose and lignin. Cellullose and hemicellulose can then be hydrolysed to obtain sugars as raw material for diverse microbial processes. Lignin can be further converted by chemical means, or it could be burned to produce heat energy. In addition, the engineering of microorganisms able to convert lignin into useful chemicals is envisaged.

The classical corn wet milling industry is another example for the basis of the biorefinery concept. In a wet mill refinery the corn is separated into corn oil (made from the germ), a fibre fraction sold as animal feed, a gluten fraction sold as protein-rich feed supplement, and finally the starch fraction that is further converted into a variety of high-value products. For instance, the starch can be liquefied and fed into a microbial process such as the production of an organic acid.

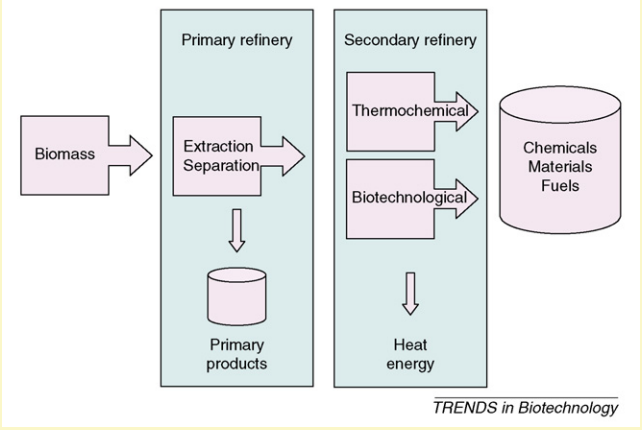

Figure I. Schematic representation of a biorefinery.

the process remain unknown. These mechanisms are currently undergoing investigation to enable improvement of the citric acid production process, for which significant improvement is no longer possible through traditional means, such as mutagenesis or cultivation optimization.

In addition to the well-established filamentous fungal species, the yeast *Yarrowia lipolytica*, has been developed as a microbial cell factory for citric acid. The starting point for this line of research was to gain access to n-paraffins [5] and fatty acids (as animal fats) [6] that are not converted by *A. niger* as carbon sources. However, *Y. lipolytica* also proved efficient in the production of citric acid from other carbon sources, such as glucose and sucrose. Citric acid concentrations of 140 g/L are now easily reached [7], and *Y. lipolytica* is probably used on an industrial scale, although few details are known of actual production methods [8].

From yoghurt to yoghurt containers: lactic acid

Lactic acid and its production by lactic acid bacteria have a long history in the food industry and microbial processes for lactic acid production were established early in the past century [9]. However, the large-scale commercial production of the purified acid by microorganisms is relatively new. The production of the biodegradable plastic polylactide (used, for instance, in food containers) led to increased interest in optically pure lactic acid. This accounts for the recent shift from chemical to microbial production processes. Approximately 150 000 tons of lactic acid were produced in 2002*, 90% of which was by fermentation with lactic acid bacteria [10].

Lactic acid bacteria have complex nutrient requirements and they ferment sugars via different pathways, resulting in homo-, hetero-, or mixed acid fermentation [10].

However, it is not only bacteria that accumulate lactate. The filamentous fungus *Rhizopus oryzae* is another natural producer [9,11,12] that has the advantage of growing on mineral medium and carbon sources such as starch or xylose.

Amber of modern times: succinic acid

The market for succinic (amber) acid is currently small and ~16 000 tons per year*. However, if the price becomes competitive, succinic acid could replace petroleum-derived maleic anhydride, which has a market volume of 213 000 tons per year. An even higher market volume is conceivable for succinic acid as it is a versatile building-block chemical suitable for many uses (Box 2). Replacing petroleum-derived chemicals, and taking into account that succinic acid formation consumes CO_2 (theoretically 1 mole CO_2 per mole succinic acid produced) the introduction of succinic acid as a commodity building-block has the potential to lead to large reductions in environmental pollution. To date, no industrial process for microbial succinic acid production has been established; however, calculations show that such a process can be competitive [13] provided that some of the issues outlined below can be resolved.

The first approach for microbial production of succinic acid was the engineering of the mixed acid fermentation of *Escherichia coli* [14]. Later it was discovered that several anaerobic rumen bacteria naturally produce large amounts of succinic acid. However, cultivation of such bacteria is dependent on expensive and complex nutrient sources, and by-product formation is a general problem that remains to be solved (reviewed by Song and Lee [13]).

Another interesting strategy was the engineering of a recombinant *E. coli* by a systems biology approach using a comparison to *Mannheimia succiniciproducens* [15], a rumen microorganism, isolated from a Korean cow. However, the yield of succinic acid in the resulting strain was significantly lower than that of the natural producers.

Finally, filamentous fungi, including *Penicillium simplicissimum*, have been shown to accumulate succinic acid naturally [16]. Although the titers and yields are also not

* Patel, M. *et al.* (2006) Medium and Long-Term Opportunities and Risks of the Biotechnological Production of Bulk Chemicals from Renewable Resources – The Potential of White Biotechnology: The BREW Project (http://www.chem.uu.nl/brew/).

Table 1. Organic acids as high-volume products[a]

Number of carbon atoms	Organic acid	Annual production (t)	Annual production by microbial process (t)[b]	Projected market volume (t)	Use (examples)
C_2	Acetic acid	7 000 000	190 000		Vinylacetate for polymers, ethylacetate as 'green' solvent
C_2	Oxalic acid	124 000	–		Synthetic intermediate, complexing agent
C_3	Acrylic acid	4 200 000	–		Polymer production
C_3	3-hydroxypropionic acid	n.a.	–	Up to 3 600 000	Potential substitute for acrylic acid and production of biodegradable polymers
C_3	Lactic acid	150 000	150 000		Food and beverages, biodegradable polymer production
C_3	Propionic acid	130 000	n.a.		Food and feed
C_4	Butyric acid	50 000	n.a.		Therapeutics, aroma, fragrance
C_4	Fumaric acid	12 000	–	>200 000	Food and feed, polyester resins
C_4	Malic acid	10 000	–	>200 000	Potential to replace maleic anhydride
C_4	Succinic acid	16 000	–	>270 000	Potential to replace maleic anhydride, manufacture of tetrahydrofuran, polymers
C_5	Itaconic acid	15 000	15 000		Specialty monomer
C_5	Levulinic acid	450	–	High	Possible precursor for bulk chemicals
C_6	Adipic acid	2 500 000	–		Production of nylon 6,6 esters used as plasticizers and lubricants
C_6	Ascorbic acid	80 000	–		Food additive
C_6	Citric acid	1 600 000	1 600 000		Food additive
C_6	Glucaric acid	n.a.	–	High	Production of new nylons, new building-block
C_6	Gluconic acid	87 000	87 000		Food additive, metal chelator

Abbreviation: n.a., no data are available.

[a]These numbers are intended to give the reader an impression of the order of magnitude in which relevant acids are on the market. One should be aware that reliable market data are not often found in the public domain.

[b]The '–' indicates that these acids are not microbially produced on an industrial level to our knowledge.

Box 2. Building-block chemicals: the LEGO® of the chemical industry

Shifting our society's dependence from petroleum to renewable biomass does not mean that the chemical industry will loose importance. Biorefineries will not be able to directly provide every required material, but they can be used to deliver the feedstock for a sustainable chemical industry. Today's task is the development of useful building-block chemicals that can be produced from biomass and subsequently converted to several high-value chemicals and materials. Building-block chemicals are molecules with multiple functional groups that can be transformed into new families of usable molecules. Organic acids are becoming increasingly important in this respect (see Table 1). Of the twelve sugar-derived building-block chemicals identified by the US Department of Energy, nine are organic acids [2].

Most of these building-block chemicals are new in the sense that they are not currently used in chemical processes because of their high costs. Their low-cost availability is an obvious pre-requisite for the chemical industry for these substances to be considered as starting materials. Nevertheless, it is anticipated that as soon as such new building-block chemicals can be provided at a sufficiently low price, their market potential might rise instantly and significantly. One example of such a chemical is succinic acid. Succinic acid is used as a surfactant, detergent or foaming agent, as an ion chelator, and also in the food industry (as an acidulant, flavoring agent or anti-microbial agent) as well as in health-related products (such pharmaceuticals and antibiotics) [57]. Currently it is produced from petrol and is too expensive to be used as a general building-block chemical. However, provided that its price becomes competitive, succinic acid could replace petrol-derived maleic anhydride in chemical synthesis processes in the future. Some of the substances that can be derived from succinic acid are shown in Figure I. Butanediol, tetrahydrofuran and γ-butyrolactone, for instance, are standard substances for the chemical industry and can be readily obtained from succinic acid. These are used as solvents, as well as for fiber and polymer production. Succinic acid can also be polymerized directly to form the biodegradable aliphatic polyester bionolle. Dimethylsuccinate is one of the so-called dibasic esters that have great potential as solvents with environmentally benign characteristics. Thus, the potential market volume for succinic acid is high, fuelling substantial efforts to establish a microbial process for succinic acid production.

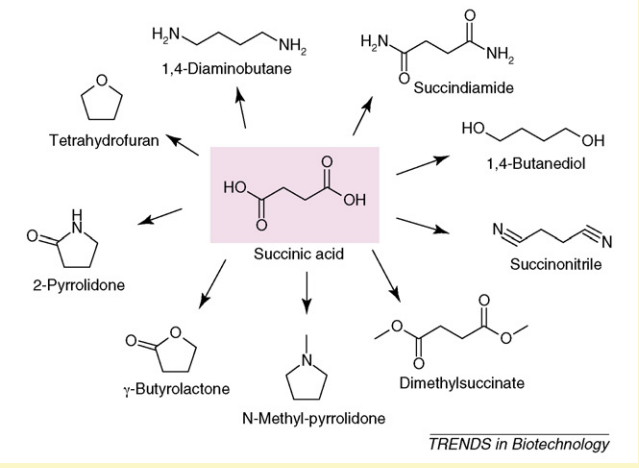

Figure I. Various substances that can be derived from succinic acid by chemical conversion.

comparable with bacterial hosts, the cultivation of the fungus is easier.

Processes at different stages: from infancy to maturity

The three examples presented are in different stages of industrial development. Hence, the questions that need to be addressed are somewhat different. An overview of the developments and the remaining obstacles, particularly in view of industrial needs, will help to draw conclusions on how to proceed in the production of organic acids with microorganisms in the future.

Citric and lactic acid production is currently based on organisms producing these acids naturally. Proper strain selection and the development of optimized bio-reactions

Table 2. Current data for citric, lactic and succinic acid production[a]

Concentration (g/L)	Productivity g/(Lh)	Yield (g/g)	Carbon source	Organism	Cultivation	Refs
Citric acid						
200			Glucose	*Aspergillus niger*		[64]
113.5		0.71	Beet, cane molasse	*Aspergillus niger*	Shake flask	[29]
114	0.61	0.76	Cane molasse	*Aspergillus niger*		[27]
40	0.1	0.99	n-Paraffin	*Yarrowia lipolytica*		[5]
42.9		0.56	Fatty acid, glucose	*Yarrowia lipolytica*		[6]
140	0.73	0.82	Sucrose	*Yarrowia lipolytica*		[7]
Lactic acid						
771	5.4			*Lactobacillus rhamnosus*	Continous product extraction	[10]
	52–144			*Lactobacillus spp.*	Cell-recycle	[10]
		3.1	Whey	*Lactobacillus spp.*	Electrodialyis	[10]
29		0.78	Red lentil flour	*Lactobacillus amylophilus*		[17]
40[b]	0.63[b]	0.95[b]	Cassava bagasse	*Lactobacillus delbrueckii*	Immobilzation and recycling	[21]
58	0.6	0.58	Xylose	*Pichia stipitis*	Hemicellulose-derived sugar	[24]
51.7		0.68	Oat	*Rhizopus oryzae*	Biorefinery appproach	[11]
95	2.2	0.80	Glucose	*Rhizopus spp.*	Airlift, industrial scale	[40]
122		0.61	Cane juice	*Saccharomyces cerevisiae*	Neutralized culture	[46]
70		0.93	Glucose	*Saccharomyces cerevisiae*	Low pH	[48]
Succinic acid						
35.6[b]	1.01[b]	0.82[b]	Wheat	*Actinobacillus succinogenes*	Biorefining strategy	[25]
52	1.8	0.76	Glucose	*Mannheimia succiniciproducens*		[49]
43	0.72	0.53	Glucose	*Escherichia coli*	Aerobic batch	[66]
58.3	1.08	0.62	Glucose	*Escherichia coli*	Aerobic fed-batch	[66]
55.2	1.15		Cane molasse	*Actinobacillus succinogenes*	Inexpensive medium	[67]
83	10.4	0.89	Glucose	*Anaerobiospirillum succiniciproducens*	Electrodialysis	[39]

[a]The data have been taken from the cited publications. If the publication has not given a parameter, no data have been given.
[b]Denotes optimal values of different cultures reported in the corresponding publication.

led to industrially viable processes. Further improvements are only possible after thorough physiological analysis of the bio-catalysts. The main focus for succinic acid production also relates to natural producers identified recently. However, the research focus is directed to systems biology and metabolic engineering because bioprocess engineering and strain selection have not proved successful.

In the following section, we outline the recent developments for the production of these three acids in the order of the industrial process (i.e starting with the substrate) using the cellular physiology in a bio-process, followed by purification. The final section deals with metabolic engineering and how this should be exploited for industrial applications.

Going large-scale: requirements for industrial scale-up

For evaluation of microbial large-scale processes the characteristics that traditionally describe the efficiency of an industrial process, namely yield, product concentration and productivity, are not sufficient. The costs of substrate and downstream processing are crucial constraints for a process to become economically viable. This is particularly true for commodity chemicals for which the price might be only slightly higher than the price for refined sugar.

In fact, a bio-process optimized for yield, product concentration and/or productivity as established in academia does not necessarily represent the most favorable situation when viewed from an economic standpoint. High substrate costs can abolish the advantage of high product concentrations, or high purification costs can absorb any possible cost advantages of an inexpensive carbon source. Clearly, these factors are interrelated. For example, refined sugar is expensive as a carbon source, but purification of the produced acid might be much easier and cheaper. On the other hand, natural carbon sources as outlined below are significantly cheaper than refined sugar (depending on the context, they might even be free to the producer), but they comprise a large amount of substances that might interfere with the bioprocess and that have to be eliminated during downstream-processing, which adds to the final costs.

Starting cheap – ending cheap? The proper choice of substrate is crucial

To improve the established processes of citric and lactic acid production access to cheap substrates is becoming crucial. Examples of local and inexpensive carbon sources reported for production of lactic acid include red lentil flour in India [17,18], kitchen waste in Japan [19], barley hydrolysates in the EU [20] and oat [11] or liquefied cornstarch from cassava bagasse [21,22].

A large amount of research is dedicated to lignocellulosic biomass. This is highly abundant, making it an interesting substrate for microbial production processes in general. However, few microorganisms can metabolize the pentoses derived from these raw materials, so this type of biomass has not been used extensively to date. The fungus *R. oryzae* and the Gram-positive bacterium *Bacillus coagulans* have been shown to convert xylose or hemicellulose hydrolysates, respectively, to lactic acid [12,23]. The yeast *Pichia stipitis* is also a promising biocatalyst capable of fermenting xylose, but it does not naturally produce lactic acid. Ilmen *et al.* report the metabolic engineering of *P. stipitis* to enable this yeast to produce lactic acid [24].

If a substrate that no organism can convert directly to the desired product is to be used, two-step biorefinery

Box 3. Transport processes are crucial for microbial organic acid production

Microbial production of organic acids involves various transport processes, such as substrate uptake, product export and possibly transport between organelles. These processes are fundamental to the microbial production of organic acids.

However, the mechanisms of most of the transport processes, and particularly the question of how much energy these processes demand, remain an area of lively scientific debate [4,58–61].

It has been pointed out for citric acid production with *A. niger* that glucose uptake energetically resembles simple diffusion [32]. Some glucose transporters of this fungus have been characterized. However, none of them show the properties determined for the uptake of glucose during citric acid production, suggesting that there might be others that have not yet been identified. Because the genomic sequence of *A. niger* has been published recently [62], this question might be addressed soon. For *S. cerevisiae* it has been shown that an increase in sugar uptake can result in an increase in productivity [63].

The question of how microbially produced acids are exported is particularly controversial [4]. Considering the significant gradient that has to be overcome to accumulate, for example, up to 200 g/L [64] of citric acid at low pH in the culture broth, an efficient transport system must be present. How is it constituted and does it need energy? It has been reported that citric acid is exported by an ATP-dependent process [65]. By contrast, Burgstaller suggests that citric acid could be excreted by passive diffusion [59]. He points out that the low pH of the culture broth is the driving force. At low pH the concentration of the deprotonated citric acid anion is low outside the cell, but high inside owing to the higher intracellular pH. Hence, this ion could leave the cell by passive diffusion. However, how the protons are exported against the gradient remains unclear.

Van Maris *et al.* set out to calculate the energy demand of lactic acid export at different extracellular pH values [61]. They point out that under typical production conditions the export of the acid necessarily involves energy consumption. They also showed that recombinant homofermentative lactate-producing *S. cerevisiae* cells require oxygen for the generation of ATP [60]. They conclude that the export of the acid is therefore energy-requiring.

The take-home massage is that transport phenomena, such as substrate uptake and product export, are important factors to be examined for improving production processes. First steps are described above, but further investigation will be necessary to gain in-depth insight in this area.

approaches become attractive. The inexpensive carbon source is first converted into a stream of nutrients readily accessible to a variety of microorganisms. This nutrient stream is then converted into the desired product in a second step. This has been suggested for succinic acid production [25]. In the first step, wheat flour is converted into a generic microbial feed-stock by a fungal bioprocess. In the second stage, the generic feedstock is converted into succinic acid by bacterial fermentation with *Actinobacillus succinogenes*. The wheat hydrolysate can substitute for refined glucose, and a fungal autolysate is a substitute for yeast extract or other costly nitrogen sources. However, at this stage an optimization of the medium is required because the fully wheat-based medium does not lead to the same fermentation characteristics as the semi-synthetic medium.

In the case of citric acid production with *A. niger* the sensitivity of the process to trace metals and other impurities limits the use of inexpensive carbon and nitrogen sources significantly. The exact physiological basis for this sensitivity is not entirely clear. Consequently, strain selection and subsequent systematic testing [26–29] are currently required to enable the use of inexpensive carbon sources. This highlights the fact that a thorough physiological analysis of the microorganism is essential for improving an established industrial process.

How physiology leads the way to yield

How physiological analysis can lead the way to improving an industrial process is best exemplified with the case of citric acid. The long-established fungal production process of citric acid seems difficult to improve by traditional means such as strain mutagenesis or bio-process engineering. But are there really no improvements possible?

Alvarez-Vasquez presented an extensive mathematical model of the metabolism of *A. niger* as a rational basis for the optimization of citric acid production [30]. The model predicts room for a fivefold increase of the citric acid production rate, even keeping the overall concentration of enzymes constant. So, why is it so hard to improve the strains by mutagenesis? The answer is that a minimum of 13 enzymatic activities must be modulated before a significant increase in citric acid production rate can be observed. Transport processes, such as sugar uptake, citric acid excretion and transport between the cytosol and the mitochondrion, are prominent metabolic activities to be modulated. In fact, these transport processes are emerging as a crucial bottleneck for microbial organic acid production in general. Box 3 outlines some current findings in this regard.

In addition, in *A. niger* citric acid is a potent inhibitor of glycolysis, which is one of the major metabolic pathways for citric acid accumulation (Figure 1). Citric acid effectively inhibits phosphofructokinase I (pfk). Until recently it was generally accepted that a certain intracellular ammonium concentration would relieve this inhibition [4]. However, Papagianni showed that the intracellular ammonium concentration is low throughout the citric acid production process [31]. How is citric acid production possible at all, then?

Legisa and Mattey describe post-translational modifications of pfk I triggered by a drop of the intracellular pH and a cAMP peak before the onset of citric acid accumulation [32]. The enzyme is cleaved and phosphorylated leading to a fragment that is highly active and highly inducible by ammonium, but less susceptible to citrate inhibition, which explains why glycolysis is not repressed.

The findings described so far relate directly to metabolic pathways and enzymatic activities. However, the correct morphological appearance of the cells is also a crucial prerequisite for efficient organic acid production [3,9,33]. Process optimization often centers on the issue of fungal morphology. Growth in the form of pellets of $<\sim$1 mm in diameter is associated with high production rates and yields [9]. To date the conditions for obtaining such pellets have been determined empirically [34,35]. The regulating signals involved remain unclear, but investigations are necessary and ongoing [36].

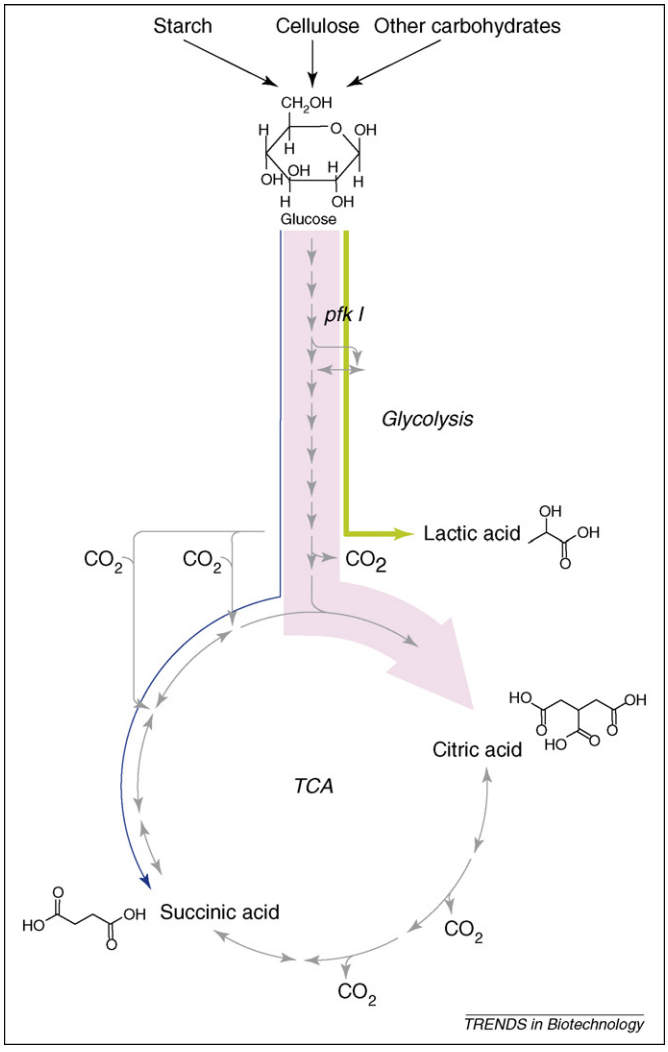

Figure 1. Schematic representation of the metabolic pathways from glucose to citric acid, lactic acid and succinic acid. For clarity, only the major pathways are depicted. Each grey arrow represents one enzymatic conversion. The size of the colored arrows represents the current annual production of each acid. Abbreviations: pfk I, phosphofructokinase I; TCA, tricarboxylic acid cycle.

Bio-process optimization: technical support of natural abilities

To gain the most from the producing organism, the process must be optimized taking into account biological as well as economic constraints. Viewing the bioprocess objectively, it appears that biomass accumulation somehow wastes the carbon source. Instead of being converted into product, the substrate is converted into the catalyst. Many studies are therefore related to the idea of uncoupling biomass accumulation from production.

One approach is the immobilization of the microbial cells to facilitate their reuse. Published examples include lactic acid production by pectate-entrapped lactobacilli [37], or repeated fed-batch fermentations with immobilized lactobacilli [38].

Another example is the production of succinic acid with the natural producer *Anaerobiospirillum succiniciproducens* in a cell-recycle bioreactor [39]. High succinic acid concentrations and high productivity were obtained when the product was continuously removed – high product concentrations seem to be deleterious for the cells. However, whether these types of complex processes are applicable on an industrial scale remains an open question. Industry demand points in the opposite direction, that is to put in place less sophisticated techniques. For instance, Liu *et al.* successfully used airlift bioreactors for a scale-up of lactic acid production with *R. oryzae* [40]. Airlift bioreactors are a low price alternative to stirred tank reactors and could potentially have an important role in microbial bulk production processes in the future. However, academia does not always seem to be concerned with the actual needs of the industry, but rather focuses on single parameters, such as high yields or high titers, ignoring the costs for obtaining them.

Considering the extremely low prices required for bulk chemicals to enter the market, investments in equipment, as well as in the operating expenses of the industrial production process, need to be extremely low. This therefore excludes the use of complex equipment and process steps. At the same time it is precisely this area in which it is almost impossible to get information from industry because the data are highly sensitive and are not being published. Consequently, academic science in this area is often based on assumptions or scientific interest and not necessarily on market facts.

Starting cheap – ending cheap? Purification of the acid adds to the production costs

An integral part of process optimization must be the reduction of the purification costs, but as outlined in the previous section, these are highly interconnected with biological and economic factors. Purification costs are higher with less purified substrates and by-products can also constitute an important problem.

A major product from the commodity chemical lactic acid is polylactide for use as packaging material [41,42]. Because the price for packaging materials is necessarily low, the lactic acid produced must also be inexpensive. At the same time the quality of the polymerized product strongly depends on the purity of the acid [42,43], requiring extensive purification steps. The optical purity of the acid and in particular the absence of di-acids (such as succinic acid, a typical by-product of bacterial fermentations) are crucial for proper polymerization. Several approaches can be used for separation of lactic acid from the culture broth, including extraction using solvents, ion-exchange separation, vacuum distillation and membrane separation [42]. However, to ensure that minimum effort is required for purification, the preceding bio-process should avoid accumulation of impurities as much as possible from the beginning of the process.

In the production of lactic acid by lactic acid bacteria, three problems can be identified that lead to increased purification costs:

(i) The strict physiological demand to keep the pH of the culture broth between 5 and 7.
(ii) The requirement for complex media components such as yeast extract, leading to a large amount of impurities in the broth.
(iii) The accumulation of organic by-products, such as succinic acid.

The demand for the high pH during fermentation requires large amounts of base. Furthermore, the free lactic acid is the desired product, so at the end of the process the broth must be acidified. Usually, the counter-ions of the base used during cultivation and the acid form large amounts of precipitated salts, which are costly to dispose of.

One approach for solving this problem is the adaptation of lactobacilli to low pH. Patnaik and co-workers set out to improve the acid tolerance of an industrially relevant *Lactobacillus* strain by genome shuffling [44]. They increased lactic acid accumulation at pH 4 by a factor of three – a promising result. Nevertheless, this pH is still not low enough to produce mainly the acid rather than its salt.

Another approach is the use of bakers yeast (*Saccharomyces cerevisiae*) as an alternative biocatalyst for lactic acid production. It grows in mineral medium and naturally tolerates low pH [45], which addresses the first two problems. However, *S. cerevisiae* ferments glucose to ethanol instead of lactic acid. By metabolic engineering the carbon flux has been successfully redirected from ethanol to lactic acid production [45–47], leading to a process based on mineral medium with a final pH lower than 2.5 [48]. The significant reduction of the purification costs makes this approach interesting, even if the final product concentrations are lower than those obtained by lactic acid bacteria.

Integrated purification systems such as electro-dialysis or certain membrane techniques could also solve some of these problems [10,13,39]. However, these techniques are expensive and, as outlined previously, it remains questionable whether they are feasible for industrial large-scale production processes [10].

The final problem of by-product formation can only be solved by metabolic engineering. Although it has not been approached up to now for lactobacilli, to the best of our knowledge this is the approach of choice for succinic acid production.

For example, *M. succiniciproducens* accumulates lactic acid, acetic acid and ethanol, in addition to succinic acid. Metabolic engineering in combination with a precise definition of the culture conditions have been used to increase productivity and to reduce by-product formation [49,50], thereby reducing purification costs suggesting that an industrial production process is closer than ever.

Metabolic engineering: adaptation of microorganisms to meet technical constraints

As outlined previously, there are problems that simply cannot be solved by proper selection of the production organism and bio-process engineering. The organisms themselves must be altered in a rational way to be able to cope with the constraints of cost effective production – metabolic engineering is the required tool.

Inhibition of by-product formation is an example that is particularly relevant for succinic acid production [49,50]. Another example is the prevention of oxalic acid accumulation during citric acid production with *A. niger* [51].

However, although the modification of defined pathways and the simple production or avoidance of metabolites in an organism is usually straightforward, it should be recognized that only a few of these approaches have real industrial applications. The majority of metabolic engineering processes fail during the scale-up phase. One of the reasons is exposure of the bio-catalyst to a variety of stresses. Stress requires the cell to dedicate more effort to maintaining its natural equilibrium. This greater effort leads to several consequences, including a change in metabolic activity, lower growth rate, lower viability and lower productivity, to name a few. Strain robustness, which is the ability of the microorganism to withstand the production environment, is therefore a key factor determining whether a microbial process will be successful and industrially viable. For instance, the concentration of lactic acid produced by recombinant *S. cerevisiae* at low pH seems still to be limited by the ability of the yeast cells to survive the harsh production conditions. A promising metabolic engineering approach aimed to improve strain robustness reducing the production of reactive oxygen species (ROS). ROS are central players involved in cellular stress, damaging the cell and ultimately leading to its death. Recombinant production of ascorbic acid by metabolic engineering led to a decrease of ROS and increased viability of stressed yeast cells [52] suggesting a new and important direction for process optimization by metabolic engineering.

Conclusion: the stressful path from nature to industry

Taking these findings together it becomes evident that the quickest way to establish an industrial process for microbial organic acid production is the exploitation of natural producers with thorough bio-process engineering. A careful consideration of the biodiversity of species is generally advisable – for the majority of small organic acids a natural producer might exist.

Metabolic engineering helps to solve defined problems, for example limiting by-product formation or broadening the range of carbon sources used (for a recent review see Kern *et al.* [53]). However, our focus in the future should move towards influencing complex cellular processes and properties, such as transport and morphology, because these are inherently connected with process performance, however we do not know enough yet for rational modification in these areas.

An increase in strain robustness (or stress resistance) will be another key field of research because cost reduction is still the main focus if new microbial organic acid production processes are to become industrially viable. The problem is not that we cannot produce a variety of acids yet, but rather that the high costs of these processes prevent large-scale production. By increasing stress resistance of the microorganisms involved the process costs can be reduced appropriately.

An integrated picture of the process is currently missing within the scientific literature. Academic research is often dedicated to isolated problems and tends to ignore the greater context of industrial manufacturing.

The choice of organism influences by-product formation and therefore influences costs. Strain robustness inevitably has to be considered for a process to be viable at large scale. The choice of the carbon source cannot be made without thinking about the purification. We therefore rely on, and request industry to better communicate their needs to ensure that public money spent in academic

institutions is invested in a useful manner and not dedicated to problems that are far from reality.

The steps covered in this article are small aspects of microbial organic acid production as a whole, but taking them into account, the road leading to a sustainable society becomes more and more visible. We agree with Hermann and Patel in concluding that a large number of white biotech products are already economically viable compared with their petrochemical equivalents [54], and we feel that organic acids constitute a class of molecules with a great future ahead of them.

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