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Organic Acid Production by Filamentous Fungi

Jon K. Magnuson and Linda L. Lasure

1. Introduction

Many of the commercial production processes for organic acids are excellent examples of fungal biotechnology. However, unlike penicillin, the organic acids have had a less visible impact on human well-being. Indeed, organic acid fermentations are often not even identified as fungal bioprocesses, having been overshadowed by the successful deployment of the β-lactam processes. Yet, in terms of productivity, fungal organic acid processes may be the best examples of all. For example, commercial processes using *Aspergillus niger* in aerated stirred-tank-reactors can convert glucose to citric acid with greater than 80% efficiency and at final concentrations in hundreds of grams per liter. Surprisingly, this phenomenal productivity has been the object of relatively few research programs. Perhaps a greater understanding of this extraordinary capacity of filamentous fungi to produce organic acids in high concentrations will allow greater exploitation of these organisms via application of new knowledge in this era of genomics-based biotechnology.

In this chapter, we will explore the biochemistry and modern genetic aspects of the current and potential commercial processes for making organic acids. The organisms involved, with a few exceptions, are filamentous fungi, and this review is limited to that group. Although yeasts including *Saccharomyces cerevisiae*, species of *Rhodotorula, Pichia*, and *Hansenula* are important organisms in fungal biotechnology, they have not been significant for commercial organic acid production, with one exception. The yeast, *Yarrowia lipolytica*, and related yeast species, may be in use commercially to produce citric acid (Lopez-Garcia, 2002). Furthermore, in the near future engineered yeasts may provide new commercial processes to make lactic acid (Porro *et al*. 2002).

This chapter is divided into two parts. The first contains a review of the commercial aspects of current and potential large-scale processes for fungal organic acid production. The second presents a detailed review of current knowledge of the biochemistry and genetic regulation of organic acid biosynthesis. The organic acids considered are limited

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Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine. Edited by Jan and Lene Lange, Kluwer Academic/Plenum Publishers, 2004.

to polyfunctional acids containing one or more carboxyl groups, hydroxyl groups, or both, that are closely tied to central metabolic pathways. A major objective of the review is to link the biochemistry of organic acid production to the available genomic data.

2. Commercial Successes: Organic Acids from Filamentous Fungi

Although many organic acids are made by living cells, few are produced commercially. Citric, gluconic, itaconic, and lactic acids are manufactured via large-scale bioprocesses. Oxalic, fumaric, and malic acids can be made through fungal bioprocesses, but the market demand is small, since competing chemical conversion routes are currently more economical. A few other organic acids have been explored for the development of novel processes. To date, the largest commercial quantities of fungal organic acids are citric acid and gluconic acid, both of which are prepared by fermentation of glucose or sucrose by *A. niger*. Another *Aspergillus* species, *A. terreus*, is used to make itaconic acid. A significant commercial source of lactic acid at the time of this writing is a bioprocess employing the Zygomycete fungus *Rhizopus oryzae*.

These three species of fungi were initially chosen for process development because they exhibited the ability to produce large amounts of a particular organic acid. This prompts us to ask why these fungi produce seemingly ridiculous quantities of organic acids. One could reasonably argue that in their natural habitats (mostly soils) these fungi would not encounter high concentrations of free sugars very frequently and, consequently, may not have evolved tight regulation of acid production. Thus, when placed in an artificial medium with high carbohydrate, they may engage in profligate acid production, ultimately resulting in their own demise. Alternately, one might argue that the ability to acidify their environment confers a competitive advantage on these fungi. First, the chelating properties of citric acid in conjunction with the increased solubility of most metal compounds at acidic pH may allow *A. niger* to grow in environments where metals are present at very low concentrations or in an insoluble state. Second, acidification would inhibit the growth of competitors, as a majority of rapidly growing bacterial species and many fungi cannot grow below pH 3.

The three fungal species produce a variety of organic acids, which may reflect different strategies to compete with other microorganisms. Many strains of *A. niger* can lower the pH of their environment by oxidizing glucose outside the cell wall, converting it to gluconic acid via the action of the enzyme glucose oxidase. The ability to catabolize gluconic acid is more unusual than the catabolism of glucose, and gluconic acid is also an effective chelator and acidulant. Other strains of *A. niger* produce citric acid intracellularly and export the acid, perhaps as a chelator and acidifier that can also be reabsorbed for use as a carbon source. *A. terreus* acidifies the environment by producing itaconic acid. Itaconic acid is not a primary metabolite, so both the anabolism and catabolism of this acid are relatively rare metabolic attributes. Once again, acidification of the environment with itaconic acid will inhibit the growth of many microorganisms. Subsequently, the relatively unusual nature of itaconic acid would permit *A. terreus* and only a few other species to catabolize the acid. It is interesting to note that the aspergilli, and all the other filamentous fungi of the phylum Ascomycota fail to produce lactic acid. The ability to produce large amounts of lactic acid appears to be restricted to the phylum Zygomycota. Perhaps fungi classified

as Zygomycetes, including *R. oryzae*, have developed a different strategy for acidifying the environment by producing lactic acid to compete with fungi unable to metabolize lactic acid. These fungi often produce both ethanol and lactic acid, a combination that would discourage many competitors.

The four commercial organic acids produced by fungi are employed in high-volume, low-value applications. For example, they are used in industrial metal cleaning or other metal treatments and in the food and feed industry as flavor enhancers, acidifiers, stabilizers, or preservatives. The commercial success of fungal bioprocesses is ultimately based on rapid and economical conversion of sugars to acid, but that alone does not explain the commercial situation for each of these acids. An understanding of the economic and business parameters that have contributed to the success of these four products may be useful in development and commercialization of new organic acid products from filamentous fungi.

2.1. Citric Acid

One of the most significant parameters explaining the commercial success of citric acid is the huge market size. In 1998, the worldwide production of citric acid was 879,000 metric tons (Lopez-Garcia, 2002), a number that far exceeds the production of any other organic acid made by fermentation. About 70% of the marketed citric acid is used in diverse food and beverage products, with carbonated beverages accounting for nearly 50% of the total production in the 1990s. The remainder of the market is mostly pharmaceutical formulations, though the metal cleaning and detergent markets are expected to increase. The market size continues to grow, largely because of expanding food and beverage markets in developing countries. The selling price of citric acid has continuously decreased over the last two decades as the market shifted from pharmaceutical to food applications, and this change in the economic climate is reflected in the ownership history of citric acid manufacturing plants. From about 1950 to 1980, citric acid was used primarily in pharmaceutical or health-related consumer products. In the early 1980s, the two largest manufacturers controlling the majority of the market were Pfizer and Miles/Bayer, suppliers of prescription drugs and over-the-counter remedies. By the early 2000s, with the expansion of the market into carbonated beverages and prepared foods, nearly all the citric acid manufacture worldwide was integrated into the corn wet milling industry either by acquisition (Archer Daniels Midland bought the Pfizer business and Tate and Lyle bought the Miles/Bayer business) or new process development (Cargill). Citric acid was a logical product line addition for the corn wet milling industry since the glucose syrups prepared from corn could be used to make the two major ingredients of carbonated beverages: citric acid by fermentation and high-fructose corn syrup by an enzymatic route (immobilized glucose isomerase). Today citric acid is considered a commodity chemical and is available as dry crystals in the anhydrous or monohydrate form. There are also several grades of 50% (w/w) solutions (saturated) made either from crystalline citric acid or from citric acid process recovery streams.

In addition to fermentation productivity and yield, two other attributes of organic acid manufacture are important economic factors: recovery and formulation of the final product. Two recovery processes are approved for food-grade citric acid in the United States: lime/sulfuric acid precipitation and liquid extraction (Title 21CFR173.280, 1984; Title 21CFR184.1033, 1994). The first step in either process is the separation of the

fermentation liquor from the biomass by filtration or centrifugation (the by-product biomass can be sold as a supplement for animal feed). In the lime/sulfuric acid process the fermentation liquor is then treated with calcium hydroxide to precipitate calcium citrate, which is filtered from the slurry, washed free of impurities, and dissolved with sulfuric acid. The insoluble calcium sulfate generated is separated from the citric acid solution, and the solution is deionized and concentrated for crystallization to form either anhydrous or monohydrated citric acid. The major disadvantage of this process is the calcium sulfate by-product, which constitutes a significant disposal problem. The other approved process is counter-current liquid extraction, which employs a mixture of tri-laurylamine, n-octanol, and decane or undecane to extract citric acid from the fermentation broth, followed by extraction of the citric acid back into water at higher temperature (Baniel *et al*., 1981). Subsequent purification steps include solvent washes, passage through activated carbon, concentration by evaporation, crystallization, and drying. In an alternative patented process, citric acid is recovered from aqueous solution via anion-exchange with a tertiary amine resin followed by thermal desorption (McQuigg *et al*., 2000).

2.2. Gluconic Acid

Gluconic acid can be prepared in a bioprocess employing *A. niger*. Unlike the citric acid process where glucose is taken up by the organism, converted to citric acid, and exported, gluconic acid is produced extracellularly. Glucose in the medium is oxidized in a two-step reaction to gluconic acid through the action of glucose oxidase. The process can also be conducted in the absence of cells with glucose oxidase and catalase derived from *A. niger*, and under the appropriate conditions, nearly 100% of the glucose is converted to gluconic acid. The enzymatic process has the added advantage that no product purification steps are required. Both processes are approved by the FDA (Title 21CFR184.1318, 1986). In addition to many food applications, gluconic acid is used as an additive to improve cement hardening.

The economic parameters involved with the manufacture and marketing of this acid are complex. Gluconic acid can be a by-product of the glucose oxidase production processes, or it can be made very efficiently from glucose syrups by enzymatic conversion (Lantero and Shetty, 2001). Another common food additive, glucono-δ-lactone, can be prepared by crystallization from solutions of gluconic acid. Gluconic acid production has been an important example of a fungal bioprocess in the past, but it appears that the process may contribute a decreasing proportion of the commercial production volume as an enzyme-based process becomes cost-effective.

2.3. Itaconic Acid

A. terreus is employed for itaconic acid production in a process similar to that for citric acid. Both processes were invented about the same time (Kane 1945; Nubel and Ratajak, 1962; Batti and Schweiger, 1963), and both can be conducted in the same manufacturing facility. At this writing, the sole producer of itaconic acid in the USA also produces citric acid. Although the process is similar to that for citric acid, as would be expected for a by-product of the citric acid cycle, there is a significant difference: the sensitivity of the organism to the acid that necessitates neutralization to obtain yields above 80 g/L (Nubel and

Ratajak, 1962). Recovery of itaconic acid can be accomplished with the technology used for citric acid, and the final product can be prepared as a dry crystalline powder.

In contrast with citric, gluconic, and lactic acids, itaconic acid is used exclusively in non-food applications. Its primary application is in the polymer industry where it is employed as a co-monomer at a level of 1–5% for certain products. Itaconic acid is also important as an ingredient for the manufacture of synthetic fibers, coatings, adhesives, thickeners, and binders. The market volume has been estimated to be about 15,000 metric tons per year and is expected to grow if the selling price (estimated to be about US$4 per kg) can be reduced (Willke and Vorlop, 2001). To date very little research has been directed at the improvement of itaconic acid production. In contrast, there has been a larger research effort directed at lactic acid production to feed the market for biodegradable plastic.

2.4. L-Lactic Acid

There are several methods to prepare lactic acid. Among the biological routes is a process employing *R. oryzae*, a fungus unrelated to the aspergilli used for the other organic acids. Taxonomically *R. oryzae* belongs to a completely different phylum in the Fungi, and its strategy for acidifying the environment also appears to be distinct. The organism imports glucose and exports lactate, an acid that is not a component or by-product of the citric acid cycle. Lactate is produced by the organism aerobically, and the commercial process requires agitation and aeration just as the other fungal organic acid processes do.

The fungal lactic acid process faces several economics challenges. One dissimilarity with *A. niger* processes is that the growth and metabolic function of *R. oryzae* is inhibited below pH 4.5, and continuous neutralization of the fermentation is required to achieve the currently maximal yield of ~80 g/L. The pH sensitivity of the organism and the tendency for filamentous growth, which further complicates the process, increase manufacturing costs. Recent research efforts have been focused on discovering alternate producers or engineering lactic acid production in a more suitable microorganism (Porro *et al*., 2002). The substrate for the *R. oryzae* process is glucose, and the manufacturers are corn-processing companies with readily available low-cost glucose. Process improvement research has included cloning the lactate dehydrogenase genes and metabolic engineering (Skory, 2000, 2001).

Lactic acid is recovered by the technologies used for the other organic acids, including precipitation from an alcoholic extract. In aqueous solution, lactic acid dimerizes to form lactide, an intermediate for the biodegradable plastic, polylactic acid.

Until recently, lactic acid was used primarily in the food industry as a preservative, flavor enhancer, and acidulant. The global market has been estimated to be about 100,000 tons/year. This is expected to grow rapidly when facilities for polylactic acid manufacture become operational: a single plant scheduled for start-up in 2002 will expand the market by 30%. Another non-food application for lactic acid is the manufacture of the biodegradable solvent, ethyl lactate.

2.5. Market Prospects

Initially, it seems surprising that so few fungi are used for organic acid manufacture, given their efficiency at producing high concentrations of various acids. However, the relevant economic driver is the continuing availability of inexpensive petroleum-derived

carbon backbone molecules for chemical synthesis of organic acids. To date, the food applications market has demanded low cost organic acids. Similarly, the biodegradable polymer market demands low cost monomers. The increased demand for racemically pure lactic acid to feed the burgeoning polylactide manufacturing capability may lead to more research on lactic acid biosynthesis. Positive market pressure from the upward trend in the cost of finite petroleum resources and increased interest in the manufacture of biodegradable plastics may encourage further research into both the discovery and development of new organic acid processes as well as the refinement of known processes.

3. Biochemistry and Genetics of Organic Acid Production by Filamentous Fungi

A survey of current knowledge regarding the metabolic pathways, biochemistry, and genetics of organic acid production by filamentous fungi, and opportunities for improvement of these organisms are the objectives of the second part of this chapter.

3.1. *Aspergillus* and Organic Acid Production

The genus *Aspergillus* contains the workhorses of the fungal fermentation industry. Aspergilli have found application in the production of foods, enzymes, pharmaceuticals, and organic acids. *A. niger* is the source of three organic acids, gluconate, citrate, and oxalate, and *A. terreus* is the source of itaconate. The biochemistry and physiology of these organic acid fermentations will be treated as a group, since they share similar physiology and each product is no more than one enzymatic step from the primary pathway of D-glucose and D-fructose metabolism.

3.1.1. Citric Acid

The production of citric acid is the oldest and most thoroughly studied filamentous fungal fermentation (Currie, 1917). Many of the parameters important for a productive submerged citric acid fermentation process were determined by Shu and Johnson (1947, 1948a, b). The metabolic pathway is known, as are the fermentation conditions that result in high yields (approximately 200 g/L of citric acid from 240 g/L of glucose or sucrose) in submerged culture. The critical parameters for citric acid production by *A. niger* were defined empirically and include: high carbohydrate concentration, low but finite manganese concentrations (~10 ppb), maintenance of high dissolved oxygen, constant agitation, and low pH (Schreferl 1986; Zhang and Röhr, 2002a, b). These physical and chemical conditions are important for the adoption and maintenance of a pelleted morphology, which is also critical for citric acid production. Knowledge of these factors has enabled the development of highly efficient submerged fermentations for citric acid production. Research in the last 60 years has revealed some of the answers to why these parameters are important, but many questions about the physiological and biochemical mechanisms underlying these empirically derived fermentation conditions remain unanswered.

The production of citrate from glucose or sucrose involves a large number of enzymatic steps occurring in two different membrane-bound cellular compartments, namely,

the cytosol and the mitochondrion. Glucose is taken into the cell and converted to the three-carbon acid, pyruvate, via the glycolytic pathway in the cytosol. One molecule of pyruvate is decarboxylated with the formation of acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex and another is carboxylated to oxaloacetate in the cytosol by pyruvate carboxylase. Oxaloacetate must be transported into the mitochondrion (via malate) and condensed with acetyl-CoA to form citrate. The product is transported out of the mitochondrion and finally out of the cell. The mechanisms have been exhaustively reviewed (Kubicek and Röhr, 1986; Mattey, 1992; Ruijter *et al*., 2002). The accumulated evidence on the biochemistry of citric acid production in *A. niger* will be presented with attention to possible targets for improvement.

The metabolic pathway leading to citric acid accumulation was elucidated through radioisotopic studies carried out in the 1950s (Martin 1950; Lewis and Weinhouse, 1951; Bomstein and Johnson, 1952; Cleland and Johnson, 1954). The earliest studies, performed with $^{14}CO_2$ and ^{14}C-labeled acetate, suggested a mechanism of citrate formation involving the condensation of "active acetate" (acetyl-CoA) and oxaloacetate that were derived by the decarboxylation of pyruvate and the carboxylation of pyruvate, respectively. The study by Cleland and Johnson utilized doubly labeled D-[3,4-^{14}C]glucose under citric acid production conditions (pH 2, high oxygen), which suppressed scrambling of the label by minimizing the formation and utilization of polyol by-products. Their study verified the C_2 plus C_4 mechanism, and they also demonstrated that the citric acid cycle was essentially shut down under citric acid production conditions as "very little shifting of labels in C_4 (oxaloacetate) due to equilibration with symmetrical fumarate" occurred. Subsequent studies demonstrated the fixation of carbon dioxide (Woronick and Johnson, 1960) and the enzyme involved, pyruvate carboxylase (Bloom and Johnson, 1962). Thus, the high yields observed in the citric acid production process are possible because all six carbons of the substrate, glucose or fructose, are conserved in the six-carbon product citric acid, through the glycolytic pathway and the actions of two additional enzymes, pyruvate carboxylase and citrate synthase (Figure 12.1).

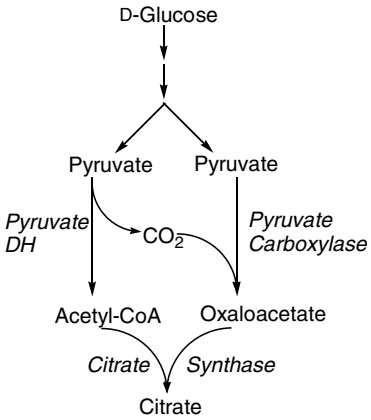

Figure 12.1. Simplified scheme of citrate synthesis in *Aspergillus niger*.

Recently, methods designed to view the citric acid production system as a whole have been applied to *A. niger*. These approaches fall under the freshly coined moniker of *systems biology*, and include metabolic modeling studies (discussed later) and NMR spectroscopy of intact cells. NMR has been used to study actively metabolizing cells of *A. niger* under various physiological conditions. Specifically, ^{13}C-NMR has been used to follow the intracellular metabolism of D-[1-^{13}C]glucose under conditions that are favorable or unfavorable for citric acid production (10% and 2% glucose, respectively) (Peksel *et al*., 2002). The labeling patterns observed were consistent with the classical ^{14}C tracer studies showing the condensation of C_2 (acetyl-CoA) and C_4 (oxaloacetate) compounds to form citrate, thus confirming the role of pyruvate carboxylase in the formation of citrate.

Many industrial citric acid fermentations use molasses as a feedstock, which is principally sucrose (α-D-glucopyranosyl-β-D-fructofuranoside), therefore, the function of invertase is important for these processes. *A. niger* hydrolyzes sucrose through the action of invertase (β-D-fructofuranosidase), about 60% of which it exports extracellularly and 40% it retains in the cytosol and/or the periplasmic space (Vainstein and Peberdy, 1991). The gene encoding this invertase, *suc1*, has been cloned and sequenced (Boddy *et al*., 1993). While low levels of invertase activity are constitutively expressed, the substrates sucrose and raffinose [α-galactosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside] are strong inducers of expression. Glucose, fructose, and xylose are strong repressors of invertase expression in the absence of sucrose but only weak repressors in the presence of sucrose (Vainstein and Peberdy, 1991). The pH optimum of the invertase encoded by *suc1* is 5.5 whereas that of the *suc2* product, a second invertase with inulinase activity is 5.0 (Boddy *et al*., 1993; Wallis *et al*., 1997). Citric acid fermentations employing *A. niger* start at about pH 3 and drop to less than pH 2, suggesting that the majority of sucrose must be imported and hydrolyzed by invertase within the cytosol. Although sucrose–proton symporters are common in the yeast genus, *Kluyveromyces* (Kilian *et al*., 1991), to our knowledge, they have not been studied in filamentous fungi.

Glucose and fructose must be transported across the cell membrane. A specific fructose transporter has not been identified in *A. niger*, but they are known in yeasts and *A. nidulans* (Mark, and Romano, 1971; Boles and Hollenberg, 1997; Heiland *et al*., 2000). Steady state kinetic analyses of intact mycelia displayed biphasic uptake of radiolabeled glucose, indicating the presence of two glucose transporters in *A. niger*; a constitutively expressed high affinity glucose transporter (K_m = 0.3 mM) and a low affinity glucose transporter (K_m = 3.7 mM), which is induced at high glucose concentrations (15%) (Torres *et al*., 1996a). Both transporters are susceptible to inhibition by citrate, although the low affinity transporter is less sensitive (Torres *et al*., 1996a). Models have been developed for both active transport and passive diffusion mechanisms based on a number of assumptions, but neither model fits the experimental data precisely (Wayman and Mattey, 2000). Nevertheless, incorporating the citrate inhibition parameters into the model suggested that the two known glucose transporters would be inadequate for the observed glucose uptake rate at the high citric acid concentrations observed during the course of an industrial fermentation. Perhaps a combination of active transport and passive diffusion occur, or an additional high affinity glucose transporter is present with the same K_m but lower sensitivity to citrate. Additional experimental studies and model refinements will be required to resolve this issue. The *A. niger* genome sequence should greatly facilitate the identification and study of additional hexose and disaccharide transporters.

Upon entry into the cytosol, glucose and fructose are phosphorylated to glucose-6-phosphate and fructose-6-phosphate, which are key branch-points for glycolysis, synthesis of intracellular storage compounds, synthesis of cell wall components, and the pentose phosphate pathway. Fructose is phosphorylated by hexokinase and glucose is phosphorylated by either glucokinase or hexokinase. The genes for both of these enzymes have been cloned and characterized from *A. niger* (Panneman *et al*., 1996, 1998). Based on the properties of the two enzymes shown in Table 12.1, it was proposed that glucokinase would account for most of the glucose phosphorylation at pH 7.5, whereas hexokinase would account for more of the phosphorylation at pH 6.5, at glucose concentrations exceeding 0.5 mM (Panneman *et al*., 1998).

^{31}P-NMR has been used to examine intact cells of *A. niger*, resulting in valuable insights about the connections between oxygen concentration, intracellular pH, and glycolytic function. The acidophilic nature of *A. niger* is demonstrated by the finding that even under the extremely low ambient pH conditions of citric acid fermentation, the cytosolic and vacuolar (mitochondrial) compartments retain pH values of 7.5 and 6.1, respectively (Hesse *et al*., 2000, 2002). Additional investigations demonstrated that intracellular pH declines as dissolved oxygen concentration falls (Legiša and Grdadolnik, 2002). Based on the kinetic parameters of the enzymes (Table 12.1), glucokinase would be expected to be responsible for a large portion of the flux from glucose to glucose-6-phosphate at pH 7.5. Nevertheless, flux of fructose or glucose through hexokinase appears to be significant during growth on sucrose greater than 5%, as disruption of the trehalose-6-phosphate synthase gene (*tpsA*) decreases the intracellular levels of the hexokinase inhibitor, trehalose-6-phosphate, and results in an increased citric acid production rate (Arisan-Atac *et al*., 1996; Wolschek and Kubicek, 1997). The investigators note that this effect would probably not be observed in a glucose fermentation; in other words, the observed effect of the *tpsA* disruption on sucrose metabolism in *A. niger* could be decreased inhibition of hexokinase leading to increasing flux from fructose to fructose-6-phosphate. The dependence of intracellular pH on oxygen concentration and the decreased specific activities of critical enzymes at acidic pH explain one facet of the physiological requirement for vigorous aeration to support high flux from glucose to citric acid.

Trehalose and the polyols, glycerol, mannitol, and erythritol, are the primary small storage molecules produced from sugars or glycolytic intermediates in *A. niger*. The accumulation and utilization of these compounds vary with culture conditions (sugar concentration) and age (Röhr *et al*., 1987; Witteveen and Visser, 1995; Peksel *et al*., 2002). The proposed physiological roles of these compounds include osmotic balance, carbon storage,

Table 12.1. Properties of Hexose Phosphorylating Enzymes in *Aspergillus niger*

Enzyme	Hexokinase	Glucokinase
EC #	2.7.1.1	2.7.1.2
pH Optimum	7.5	7.5
Activity at pH 6.5	50 %	17 %
K_m for Glucose (mM)	0.350	0.063
K_m for Fructose (mM)	2	120
K_i for Trehalose-6-P (mM)	0.01	not inhibited

redox balance, and transport through hyphae (Witteveen and Visser, 1995). During growth on 2% sucrose, glycerol and erythritol accumulate early while mannitol becomes predominant as the culture ages (Witteveen and Visser, 1995). ^{13}C-NMR studies of citric acid producing cultures growing on 10% D-[1-^{13}C]glucose indicated that trehalose and mannitol accumulated during the growth phase, trehalose declined and glycerol accumulated during the production phase, while erythritol maintained a minimal concentration throughout (Peksel *et al*., 2002). The observed labeling patterns in the polyols and the accumulation of glycerol suggest that glycolytic control is displaced from fructose-6-phosphate dehydrogenase to glyceraldehyde-3-phosphate dehydrogenase under citric acid production conditions (10% glucose media). Thus, the authors propose that glyceraldehyde-3-phosphate dehydrogenase would be a promising target for metabolic engineering.

Over the last decade, a number of glycolytic enzymes of *A. niger* have been purified, characterized, and cloned (see Table 12.2) (Habison *et al*., 1983; Meixner-Monori *et al*., 1984; Arts *et al*., 1987; Panneman *et al*., 1996; Panneman *et al*., 1998; Ruijter and Visser, 1999). Subsequently, some of these genes have been over-expressed in an effort to increase flux through this critical pathway leading to citric acid production. *A. niger* 6-phosphofructokinase (PFK), like the PFK from other organisms, is very sensitive to activation by fructose-2,6-bisphosphate (half-maximal stimulation at less than 0.2 μM) (Arts *et al*., 1987). Citrate is a strong inhibitor of PFK and the simultaneous presence of the activators AMP (0.1 mM), NH_4^+ ions (20 mM) and fructose-2,6-bisphosphate (0.2 μM) are required to overcome inhibition by citrate (5 mM) (Arts *et al*., 1987). The apparent requirement for significant intracellular NH_4^+ concentrations to relieve this inhibition may be of practical interest in the control of commercial citrate processes where NH_3 introduction is carefully metered to provide sufficient nitrogen to maintain citric acid metabolism without promoting accumulation of biomass or increasing the pH of the fermentation. Sufficient intracellular NH_4^+ may be generated due to the decreased protein synthesis and concomitant amino acid accumulation observed under the low Mn^{2+} conditions of the citric acid production process (Kubicek *et al*., 1979; Ma *et al*., 1985).

The *A. niger pfkA* gene was cloned and used to increase the expression of its product 3 to 5 times that of the wild-type strain (Ruijter *et al*., 1997). In the same study, pyruvate kinase (*pki*) was cloned and over-expressed alone or with PFK. However, in all three transformants (*pki, pfk, pki + pfk*) the citric acid production rate and the pools of glycolytic intermediates remained at the same levels observed for the wild-type strain, indicating no increase in flux through the glycolytic pathway. In the *pfkA* transformants, the concentration of fructose-2,6-bisphosphate was decreased by 40%. However, at the intracellular pH of 7.5, the levels of this PFK activator are still sufficient to keep PFK almost fully activated. Thus, some other mechanism for the control of PFK activity must be operable. One possibility is cAMP dependent kinase activation and phosphatase inactivation (Legiša and Benčina, 1994; Gradišnik-Grapulin and Legiša, 1997; Benčina, 1997). There were no significant differences between the PFK over-producers and the wild-type strain with regard to the activity levels of the other glycolytic enzymes and enzymes involved in removing glycolytic intermediates. Whatever the mechanism, it is apparent that *A. niger* carefully controls glycolytic flux even in the presence of substantial perturbations of PFK and pyruvate kinase activities.

At the terminus of glycolysis, a potential futile cycle exists involving the triad of phosphoenolpyruvate, pyruvate, and oxaloacetate catalyzed by cytosolic pyruvate kinase, pyruvate carboxylase, and PEP carboxykinase (Osmani and Scrutton, 1983; Bercovitz *et al*.,

Table 12.2. Proteins and Genes Relevant to Organic Acid Production in *Aspergillus* sp.

No.[a]	Protein	EC no.	Gene	Accession no.[b]	Organism
1	Glc[c] transporter, high aff.	n.a.	—	—	—
2	Glc transporter, low aff.	n.a.	—	—	—
3	Hexokinase	2.7.1.1	*hxkA*	AJ009973	*A. niger*
4	Glucokinase	2.7.1.2	*glkA*	X99626	*A. niger*
5	Glucose-6-P isomerase	5.3.1.9	*pgiA*	AB032269	*A. oryzae*
6	6-Phosphofructokinase	2.7.1.11	*pfkA*	Z79690	*A. niger*
7	Fructose-bisP aldolase	4.1.2.13	*fbaA*	AB032272	*A. oryzae*
8	Triosephosphate isomerase	5.3.1.1	*tpiA*	AB032273	*A. oryzae*
9	Glyceraldehyde-3-P DH	1.2.1.12	*gpdA*	Q12552	*A. niger*
10	Phosphoglycerate kinase	2.7.2.3	*pgkA*	D28484	*A. oryzae*
11	Phosphoglycerate mutase	5.4.2.1	*gpm*	X58789	yeast
12	PEP hydratase	4.2.1.11	*enoA*	D63941	*A. oryzae*
13	Pyruvate kinase	2.7.1.40	*pkiA*	S38698	*A. niger*
14	Pyruvate DH complex	1.2.4.1	—	—	—
15	Citrate synthase	4.1.3.7	*cit1*	D63376	*A. niger*
16	Citrate/malate antiporter	n.a.	—	—	—
17	Pyruvate carboxylase	6.4.1.1	*pyc*	AJ009972	*A. niger*
18	PEP carboxykinase	4.1.1.49	*acuF*	AY049067	*A. nidulans*
19	Malate dehydrogenase	1.1.1.37	—	—	*A. fumigatus*
20	Oxaloacetase	3.7.1.1	*oah*	AAA50372[d]	*A. niger*
21	Glucose oxidase	1.1.3.4	*gox*	X16061	*A. niger*
21	Glucose oxidase	1.1.3.4	*ggox*	AJ294936	*A. niger*
22	Gluconolactonase	3.1.1.17		—	—
23	Trehalose P synthase	2.4.1.15	*tspA*	U07184	*A. niger*
23	Trehalose P synthase	2.4.1.15	*tspB*	U63416	*A. niger*
24	Trehalose phosphatase	3.1.3.12	—	—	—
25	6-Phosphofructo-2-kinase	2.7.1.105	—	—	—
26	Citrate transporter, export	n.a.	—	—	—
27	Citrate transporter, uptake	n.a.	—	—	—
28	Oxalate transporter	n.a.	—	—	—
29	Aconitate hydratase	4.2.1.3	*aco*	AF093142	*A. terreus*
30	Aconitate decarboxylase	4.1.1.6	—		*A. terreus*
31	β-Fructofuranosidase	3.2.1.26	*suc1*	L06844	*A. niger*
31	β-Fructofuranosidase	3.2.1.26	*suc2*	—	*A. niger*
32	Sucrose transporter	n.a.	—	—	—
33	Fructose transporter	n.a.	—	—	—
	Mannitol-1-P DH	1.1.1.17	*mpdA*	AY081178	*A. niger*
	Isocitrate DH (NADP)	1.1.1.42	*icdA*	AB000261	*A. niger*
	Oxoglutarate DH	1.2.4.2	—	—	—
	Succinate DH (NADP)	1.3.5.1	—	—	—
	Fumarate hydratase	4.2.1.2	—	—	—
	Alternative oxidase	n.a.	*aox1*	AB046619	*A. niger*

[a]This number refers to the numbering in Figure 12.2.
[b]Accession numbers are for the GenBank/EMBL sequence databases, except [d]which is for the Derwent GENESEQ patent database.
[c]Abbreviations. Glc: D-Glucose; aff: affinity; PEP: phosphoenolpyruvate; P: phosphate; DH: dehydrogenase; n.a.: not applicable.

1990; Jaklitsch *et al*., 1991). Each turn of this cycle consumes one molecule of ATP. ^{13}C-NMR studies indicated that during the growth phase, flux from pyruvate to oxaloacetate exceeded flux from oxaloacetate to citrate (Peksel *et al*., 2002). Consistent with this observation, significant futile cycling was observed. This suggests two targets for minimizing flux through this futile cycle, decreased expression of PEP carboxykinase or increased expression of citrate synthase. Recently, a strain of *A. niger* expressing eleven times the wild-type levels of citrate synthase was successfully prepared (Ruijter *et al*., 2000). However, this strain exhibited the same rate of citrate accumulation as the wild-type strain. The other strategy, cloning and over-expression of PEP carboxykinase, has not been reported in *A. niger*. The cumulative data also suggest that increasing pyruvate carboxylase activity would simply increase futile cycling, at least in the growth phase of the fermentation.

Citrate synthase is the terminal enzyme in the citric acid biosynthetic pathway. The enzyme from *A. niger* has been characterized and cloned (Kubicek and Röhr, 1980; Kirimura *et al*., 1999a). The enzyme is inhibited by Mg^{2+} and ATP (K_i = 1 mM), but importantly, citrate is not an inhibitor of the enzyme. The results cited above showed that increasing citrate synthase expression was ineffective in increasing the citrate production rate (Ruijter *et al*., 2000). Other enzymes of the citric acid cycle have been proposed to be significant. Aconitase inhibition would theoretically lead to an accumulation of citrate by blocking subsequent flux through the citric acid cycle. However, there were no significant differences in aconitase or isocitrate dehydrogenase activity levels in the *A. niger* parent strain and in mutant strains producing higher yields of citric acid (La Nauze, 1966). Furthermore, aconitase has been shown to be active even under citric acid production conditions (Kubicek and Röhr, 1985).

Isocitrate dehydrogenase could have a deleterious effect on citric acid production by decarboxylating isocitrate, which is in equilibrium with citrate via aconitase. The $NADP^+$-specific isocitrate dehydrogenase has been purified from *A. niger* and found to be present in both the cytosol and mitochondrion (Meixner-Monori *et al*., 1986). These workers found that isocitrate dehydrogenase was inhibited by ATP and citrate via chelation of enzymatic Mg^{2+}. However, they concluded that at intracellular Mg^{2+} concentrations this would be of little significance. Recently, the *icdA* gene encoding the $NADP^+$-specific isocitrate dehydrogenase was cloned (Kirimura *et al*., 2002). A single genomic copy of this gene produces two alternate mRNA transcripts. Although the predicted amino acid sequence of one transcript encodes a mitochondrial targeting sequence, peroxisome targeting sequences are encoded in both transcripts. Decreasing the expression of this gene is a possible strategy for decreasing the loss of citrate via TCA cycling. However, the previous data suggests this approach is unlikely to have a positive effect on citric acid accumulation and may have unintended negative effects on cell viability during the growth phase.

For citric acid to accumulate extracellularly at a final concentration of about 1.0 M, an active export system must exist to remove citrate from the cytosol where the concentration is only 2–30 mM (Kubicek and Röhr, 1985; Legiša and Kidric, 1989; Prömper *et al*., 1993). Citrate export requires low Mn^{2+} concentrations in the range known to be required for efficient citric acid production (Netik *et al*., 1997). On the other hand, citrate import required Mn^{2+} both for induction of expression of the citrate importer as well as for its function (Netik *et al*., 1997). These results provide an additional explanation of the multiple effects of Mn^{2+} on the physiology of *A. niger* under citric acid production conditions.

The alternative oxidase (AOX) is an inducible component of the alternative respiratory pathway in fungi. In *A. niger*, active AOX is necessary for citric acid production and is another example of a component of *A. niger* that is sensitive to the presence of manganese

ions (Kubicek *et al*., 1980; Zehentgruber *et al*., 1980; Kirimura *et al*., 2000). In addition, AOX is apparently inactivated at low dissolved oxygen concentrations (Zehentgruber *et al*., 1980). This is one component of the observed physiological requirement for undisrupted oxygen supply to maintain citric acid production. Although the protein appears to be constitutively expressed in *A. niger*, expression is increased on transition to the citric acid production phase (Kirimura *et al*., 1987). The AOX has the desirable effect of regenerating the intracellular pool of NAD^+ by oxidizing the NADH generated in the glycolytic pathway, and it does so without excessive synthesis of ATP, since it transports only 40% as many protons as the standard respiratory chain (Joseph-Horne *et al*., 2001). Hence, only 40% as much ATP is generated by this system, which is probably sufficient under the production phase of the citric acid fermentation where little cell growth is occurring. The cDNA corresponding to the *aox1* gene of *A. niger* has been cloned, sequenced, and functionally expressed in *E. coli* (Kirimura *et al*., 1999b). Understanding the mechanism of the reportedly irreversible inactivation of AOX by low oxygen concentration could facilitate the design of a more robust *A. niger* production strain resistant to transient drops in oxygen concentration.

The net result of 60 years of work examining the biochemistry and molecular biology of citric acid production by *A. niger* has been to emphasize the exquisitely delicate control this organism exerts over its metabolic processes. Much has been learned about the pathways and the corresponding enzymes responsible for metabolism of glucose to citric acid, and the accumulated data on metabolite pool concentrations and steady state kinetic parameters of the relevant enzymes and transporters has been tabulated in support of model development (Alvarez-Vasquez *et al*., 2000). Recent modeling studies in *A. niger* and other systems have provided insights on potentially successful strategies for manipulating metabolic flux (Torres, 1994; Cornish-Bowden *et al*., 1995; Torres *et al*., 1996b; Ruijter *et al*., 1998; Alvarez-Vasquez *et al*., 2000; Guebel and Torres Darias, 2001; Peksel *et al*., 2002). The strategy of increasing the expression of an enzyme predicted to catalyze the rate-limiting step in a pathway has been calculated to be ineffective (Cornish-Bowden *et al*., 1995). This is consistent with the experimental results from genetic manipulation of the levels of expression of enzymes alone or in tandem, which have failed to increase the citrate production rate (Ruijter *et al*., 1997, 2000). It is predicted that simultaneously adjusting the expression of a large number of the enzymes in a pathway may lead to increases in metabolic flux, but this obviously raises considerable technical challenges (Cornish-Bowden *et al*., 1995; Torres *et al*., 1996b; Alvarez-Vasquez *et al*., 2000). Adjusting the step that removes the desired product is predicted by models to have the desired effect and constitutes the most promising metabolic engineering strategy due to its relative simplicity (Cornish-Bowden *et al*., 1995). This would suggest that manipulation of the transporters involved in citrate uptake and export would be a desirable strategy for increasing the rate of citrate production.

3.1.2. Oxalic Acid

Oxalic acid is produced by a wide variety of fungi, including brown-rots, white-rots, mycorrhizae, plant pathogens, and *A. niger*. In contrast to citric acid, the physiological roles of oxalic acid are well known and have been reviewed (Dutton and Evans, 1996). In *A. niger*, oxalate biosynthesis is due exclusively to the action of oxaloacetase, which catalyzes the hydrolysis of oxaloacetate to oxalate and acetate (Hayaishi *et al*., 1956; Mueller, 1975; Lenz *et al*., 1976; Kubicek *et al*., 1988). The enzyme is located in the cytosol, and expression is induced at pH values greater than 4 and by carbonate

(Kubicek *et al*., 1988; Pedersen *et al*., 2000b). The enzyme requires Mn^{2+} for activity (K_m = 21 μM), which has obvious implications for the citric acid process, and is specific for oxaloacetate (K_m = 220 μM) (Hayaishi *et al*., 1956; Lenz *et al*., 1976). The known presence of pyruvate carboxylase in the cytosol of *A. niger* (Osmani and Scrutton, 1983; Bercovitz *et al*., 1990; Jaklitsch *et al*., 1991) together with the insensitivity of oxalate production to the TCA cycle inhibitor fluorocitrate (Kubicek *et al*., 1988) indicates that oxalate is produced by a branch from the glycolytic pathway, as shown in Figure 12.2.

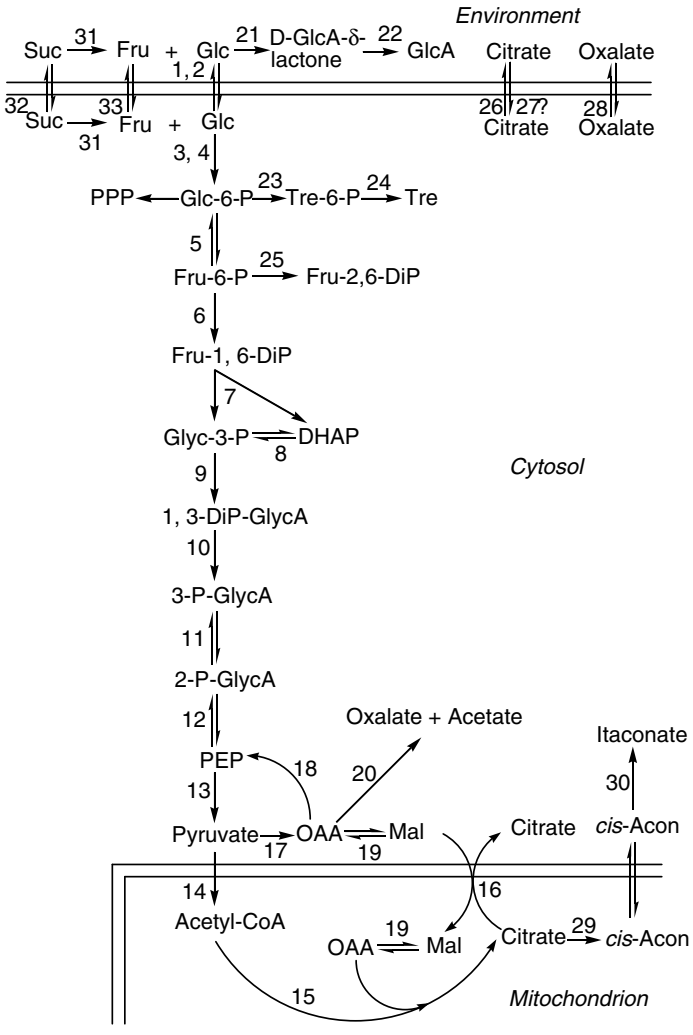

Figure 12.2. Critical pathways for organic acid synthesis in *Aspergillus* spp. Numbers refer to the proteins and genes listed in Table 12.2. Abbreviations: PPP, pentose phosphate pathway; Glc, glucose; Fru, fructose; Suc, sucrose; Tre, trehalose; GlcA, gluconic acid; P, phosphate; DiP, diphosphate; Glyc, glyceraldehydes; DHAP, dihydroxyacetone phosphate; GlycA, glyceric acid; PEP, phosphoenolpyruvate; OAA, oxaloacetic acid; Mal, malic acid; Acon, aconitic acid.

The *oah* gene has been cloned and sequenced from *A. niger* (Pedersen *et al*., 2000b), and this gene is considered necessary and probably sufficient for expression of functional oxaloacetase based on the following evidence. Disruption of the *oah* gene resulted in an *A. niger* mutant defective in oxalic acid production, demonstrating that the *oah* gene is necessary for oxaloacetase activity (Pedersen *et al*., 2000a). The relatively labile enzyme has not been purified to homogeneity, but four of the five bands observed on a denaturing polyacrylamide gel have been sequenced and demonstrated to be (proteolytic or glycosylation) variants of the polypeptide encoded by *oah*. The oxaloacetase is a large multimeric enzyme with a molecular weight of about 430 kDa, likely composed of 10–12 copies of a single polypeptide subunit of 37 kDa (Lenz *et al*., 1976; Pedersen *et al*., 2000b). Unequivocal demonstration of the sufficiency of the *oah* gene for oxaloacetase function awaits expression of the enzyme in a heterologous host. The 5′ untranslated region of the *oah* gene contains a putative FacB binding site (Pedersen *et al*., 2000b), which in *A. nidulans* is involved in control of the expression of acetate utilization genes (Todd *et al*., 1998). This is consistent with the observed maximal expression of *oah* mRNA and oxaloacetase activity during growth on acetate (Pedersen *et al*., 2000b).

The *oah* gene belongs to the isocitrate lyase family containing a conserved active site motif of K(K/R)CGH(M/L)(A/E)GK. A TBLASTN search of the *Aspergillus fumigatus* unfinished genome revealed a gene with 84% amino acid identity (91% similarity) to the translated product of the *oah* gene containing the conserved active site motif (TIGR, 2002). The putative *orf* also contained two introns in the same positions as the *A. niger oah* gene. A TBLASTN search of the *Phanerochaete chrysosporium* unfinished genome identified a putative *oah* gene bearing 48% identity (64% similarity) to the *A. niger oah* gene over the C-terminal 270 amino acids (DOE Joint Genome Institute, 2002). This Basidiomycete is known to produce and utilize oxalic acid for lignin degradation (Wariishi *et al*., 1992). These findings, though based on the limited publicly available fungal genome databases, indicate that oxaloacetase may be widely distributed in fungi, which is consistent with the widespread capability to produce oxalic acid in these organisms. However, the presence of a putative oxaloacetase does not preclude the function of the glyoxylate bypass cycle as a source of oxalate in *A. fumigatus* or *P. chrysosporium*.

Under the current economic conditions (relatively inexpensive petroleum and energy) there is little interest in producing oxalic acid by fungal fermentation but considerable interest in eliminating it as a contaminant in *A. niger* fermentations where citric acid or enzymes are the desired products. This was the goal of the study cited above where the *oah* gene was disrupted by homologous recombination in an industrial glucoamylase production strain, resulting in a transformant lacking the ability to produce oxalic acid (Pedersen *et al*., 2000a). A second study dealing with mutants derived from a citric acid producing strain of *A. niger* demonstrated that a mutant lacking oxaloacetase activity (*prtF28*) did not produce oxalic acid (Ruijter *et al*., 1999). A mutant derived from *prtF28*, which also lacked glucose oxidase activity, was able to produce citric acid at pH 5 in the presence of manganese. However, under standard citric acid production conditions (low pH) the mutant retained a sensitivity to manganese, emphasizing the multifactor nature of the manganese effect.

If the economic conditions for oxalic acid production by fungal fermentation become favorable in the future, then the accumulated information about oxalate production in *A. niger* will be useful for developing a production strain. The evidence is convincing that

oxalate production in *A. niger* is mediated exclusively by cleavage of oxaloacetate to oxalate and acetate by oxaloacetase, though the sufficiency of the *oah* gene for oxaloacetase expression remains to be demonstrated. As mentioned, the expression of the *oah* gene is induced at pH values greater than 4 and by carbonate and is additionally controlled by the acetate utilization transcription factor (FacB). Genetic engineering of an *A. niger* strain to remove these controls would be helpful in a production strain. Removal of the toxic product, oxalic acid, from the cytosol could become problematic in a production strain; as with strategies proposed for citric acid production, oxalic acid export systems may have to be up-regulated in an oxalic acid production strain.

3.1.3. Gluconic Acid

Gluconic acid production by fermentation of glucose using *A. niger* is another mature bioprocess with literature reporting highly efficient processes dating back to 1940 (Moyer *et al*., 1940; Blom *et al*., 1952). We have noted that the gluconic acid process is unique among the organic acid fermentations as it occurs entirely outside of the cytoplasmic membrane. The first step is catalyzed by glucose oxidase, which oxidizes β-D-glucopyranose to D-glucono-1,5-lactone. The hydrolysis of the lactone to form gluconic acid occurs spontaneously in aqueous solutions, but the rate is six orders of magnitude greater with the enzyme gluconolactonase (Jermyn, 1960). A partial purification of the gluconolactonase from *A. niger* has been reported (Ogawa *et al*., 2002). However, this second step is relatively unimportant from a practical standpoint, as gluconic acid, D-glucono-1,5-lactone, and D-glucono-1,4-lactone will rapidly reach equilibrium upon storage in aqueous solutions. More important is the action of catalase, which catalyzes the disproportionation of the cytotoxic hydrogen peroxide, formed by the action of glucose oxidase, into water and molecular oxygen.

The critical enzyme in this fermentation is glucose oxidase, which was first identified in *Penicillium* spp. (Coulthard *et al*., 1945). Glucose oxidase has subsequently been purified and characterized from a variety of fungi, including a variety of *Penicillium* spp. (Coulthard *et al*., 1945; Kusai *et al*., 1960), *A. niger* (Pazur, 1966), and the Basidiomycete, *P. chrysosporium* (Kelley and Reddy, 1986, 1988). The enzyme from *A. niger* is the most thoroughly studied fungal glucose oxidase, and it has been cloned, sequenced, and expressed in yeast (Frederick *et al*., 1990). It exists as a dimer of identical subunits containing one FAD per subunit. The FAD is reduced in the course of oxidizing glucose to gluconic acid, and the subsequent oxidation of the reduced FAD by molecular oxygen generates hydrogen peroxide (Gibson *et al*., 1964). Both glucose oxidase and gluconolactonase are located outside the plasma membrane and by activity staining and immunocytochemical staining glucose oxidase appears to be associated specifically with the cell wall in *A. niger* N400 (Witteveen *et al*., 1992). This is consistent with the observation that glucose oxidase from *A. niger* is glycosylated (Swoboda and Massey, 1965; O'Malley and Weaver, 1972), a general characteristic of fungal extracellular enzymes. The hydrogen peroxide generated by glucose oxidase inactivates the enzyme, probably through the oxidation of methionine residues (Kleppe, 1966). This emphasizes the need for catalase or some other mechanism of removing the hydrogen peroxide. In *A. niger*, there are two constitutive catalases and two catalases induced by dissolved oxygen concentrations of 30% or greater (Witteveen *et al*., 1992). Both the induced and constitutive pairs of catalases consist of one intracellular and one extracellular enzyme. The importance of catalase is

reflected in the design of a patented process for gluconic acid production by enzymes derived from *A. niger* where the catalase to glucose oxidase ratio is 200 or greater based on activity (Vroemen and Beverini, 1999). The identification of a large number of mutants affecting glucose oxidase production indicates that the expression of this enzyme, and gluconic acid production as a whole, is subject to complex regulation as are other organic acid production processes (Swart *et al*., 1990; Witteveen *et al*., 1990, 1993). Although gluconic acid production by fermentation of glucose with *A. niger* is in current practice, in the future it is likely that an enzymatic process will be utilized if glucose oxidase can be produced economically and in a form stable to the process conditions.

3.1.4. Itaconic Acid

The first reported biological source of itaconic acid was the descriptively named *Aspergillus itaconicus* (Kinoshita, 1931). Shortly thereafter, it was discovered that *A. terreus* produced itaconic acid (Calam *et al*., 1939). Lockwood and Reeves (1945) screened over 300 strains of *A. terreus* and found eleven that were efficient producers of itaconic acid from glucose (45% yield). Most of the work on the fermentation parameters and biochemistry of itaconic acid production has been performed with *A. terreus*, largely with strain NRRL 1960. Itaconic acid is also produced by Basidiomycetes of the genus *Ustilago* (Haskins *et al*., 1955; Guevarra and Tabuchi, 1990; Tabuchi, 1991), and a comprehensive review of itaconic acid production was recently published (Willke and Vorlop, 2001).

An efficient process for the fermentation of sucrose in molasses to itaconic acid using *A. terreus* was patented in 1962 (Nubel and Rabajak, 1962). The reported yield is 70%. In general, the parameters that are important for itaconic acid production by *A. terreus* include an incubation temperature of 37–40 °C, continuous aeration, a low starting pH (3–5), a lower operating pH (2.2–3.8), high glucose concentrations (10–20%), sufficient nitrogen, high magnesium sulfate concentration (0.5%), low phosphate to limit mycelial growth, and adequate levels of the trace metals, zinc, copper, and iron (Lockwood and Reeves, 1945; Nelson *et al*., 1952; Pfeifer *et al*., 1952; Larsen and Eimhjellen, 1955; Nubel and Ratajak, 1962; Gyamerah, 1995a, b; Willke and Vorlop, 2001). Since itaconic acid production by *A. terreus* shares many of the characteristics of citric acid production by *A. niger* it would be informative to perform a systematic survey of the effects of various trace metals in a highly defined synthetic media on fungal morphology and the production rate and yield of itaconic acid. *A. terreus* is able to grow well on a variety of monosaccharides, disaccharides, and polysaccharides but converts relatively few of these substrates to itaconic acid (Eimhjellen and Larsen, 1955). In this survey, sucrose and glucose were reported to give 57% and 52% yields on a weight basis, respectively, whereas the yields with the pentoses D-xylose and L-arabinose were only 31% and 18%, respectively (Eimhjellen and Larsen, 1955). A 45% yield of itaconic acid from D-xylose has been obtained with an immobilized *A. terreus* system growing in a 6.7% xylose medium (Kautola *et al*., 1985), but this is considerably lower than the 70% yield obtained on sucrose (Nubel and Ratajak, 1962).

The biochemical pathway resulting in itaconic acid production has been determined but the unique enzyme in this pathway, *cis*-aconitate decarboxylase, has not been purified to homogeneity (Bentley and Thiessen, 1955). Bentley and Thiessen (1957a, b, c) performed studies with ^{14}C-labelled substrates to demonstrate that the pathway for itaconic acid production in *A. terreus* paralleled that of citric acid production in *A. niger* with two

additional steps: namely, the dehydration of citrate by aconitase to form *cis*-aconitate and a decarboxylation by *cis*-aconitate decarboxylase to form itaconic acid. Aconitate decarboxylase activity was demonstrated in cell-free extracts, but its instability has been an obstacle to purification (Bentley and Thiessen, 1955, 1957c). These cell-free enzyme preparations, which contained both aconitase and *cis*-aconitate decarboxylase activities, were used to resolve the position of decarboxylation of *cis*-aconitate (Figure 12.3). Incubation with [1,6-^{14}C]citric acid resulted in the release of unlabeled CO_2, whereas incubation with D-[5,6-^{14}C]isocitric acid released labeled CO_2 (Bentley and Thiessen, 1957c). This indicated that *cis*-aconitic acid was decarboxylated at C-5. Both ^{13}C-NMR and radioisotope tracer studies with a variety of ^{13}C and ^{14}C labeled substrates have confirmed the mechanism of action of *cis*-aconitate decarboxylase and the similarity of the pathway to that of citric acid production in *A. niger* (Winskill, 1983; Bonnarme *et al*., 1995).

Cis-aconitate decarboxylase resides exclusively in the cytosol in *A. terreus* (Jaklitsch *et al*., 1991), but aconitase and citrate synthase are probably located only in the mitochondria. Therefore, *cis*-aconitate must be transported out of the mitochondrial compartment to the cytosol for decarboxylation to itaconic acid. It is not known whether *A. terreus* transports *cis*-aconitate via a specific *cis*-aconitate/malate antiporter or by the citrate/malate

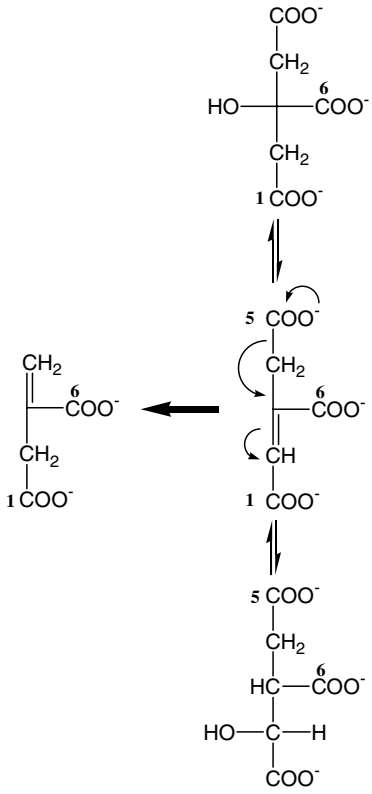

Figure 12.3. Conversion of citrate to itaconate. Labeling of carbon atoms follows that of Bentley and Thiessen (1957c).

antiporter. Work with an Egyptian isolate of *A. terreus* indicated that not only itaconic acid and citric acid but also *cis*-aconitic acid accumulated in the media (Shimi and Nour El Dein, 1962). These results indicate a need to determine the specificity of the dicarboxylic acid and tricarboxylic acid transporters of *Aspergillus* spp. Expression of *cis*-aconitate decarboxylase is induced only under itaconic acid production conditions, which also cause aconitase activity to increase 2.5– to 3.0-fold (Jaklitsch *et al*., 1991; Bonnarme *et al*., 1995).

The purification and cloning of the gene for *cis*-aconitate decarboxylase would have considerable scientific and biotechnological impact. The role of this enzyme in itaconic acid biosynthesis could be confirmed by transforming a citric acid producing strain of *A. niger*. In addition, a more efficient itaconic acid process might be devised by using a highly optimized citric acid production strain of *A. niger* as the recipient of the gene. However, the need for a specific *cis*-aconitate/malate antiporter for translocation of *cis*-aconitate from the mitochondrion to the cytosol could confound this approach. Nevertheless, such a negative result would suggest that this unique transporter is present in *A. terreus* (a somewhat circular argument). There is no theoretical reason to prevent the achievement of an itaconic acid production process that is as efficient as the citric acid production process. Genetic engineering of either *A. terreus* or *A. niger*, further refinement of fermentation conditions to obtain the optimal morphology of *A. terreus*, or the use of less expensive substrates may result in a more economical process for the production of itaconic acid.

3.2. Rhizopus and Organic Acid Production

Rhizopus spp. and related Zygomycetes (principally *R. oryzae*) are capable of producing significant amounts of L(+) lactic acid, fumaric acid, and potentially, L-malic acid. Although L-malic acid is also produced by *Aspergillus* spp. the production of these three acids will be discussed sequentially in the following section focusing on *Rhizopus* as a production organism. Generally, *Rhizopus* spp. have the desirable characteristics of growing on simple chemically-defined media, and utilizing complex carbohydrates, hexoses, and pentoses. Their main disadvantage is a tendency to produce more than one metabolic endproduct in significant yields.

3.2.1. L-Lactic Acid

The fungal production of L-lactic acid by a surface culture of *Rhizopus* spp. was reported early in the last century (Ehrlich *et al*., 1911). However, the first report of an efficient submerged fermentation for the fungal production of L-lactic acid was in 1936 (Lockwood *et al*., 1936; Ward *et al*., 1938). This was the era in which the efficiencies of submerged fungal fermentations first became widely recognized. Ward *et al*. (1938) described a fermentation process utilizing the Zygomycete genera, *Rhizopus* and *Actinomucor* in general, and *R. oryzae* (syn. *arrhizus*) specifically, which resulted in 63–69% yields of L-lactic acid from chemically defined media containing 15% glucose. They also delineated the advantages of the fungal process over the bacterial process that remain true today: the use of a chemically defined medium, including inorganic nitrogen sources, which simplifies product purification; the ability to metabolize high concentrations of glucose, thus obtaining high product concentrations; and the production of enantiomerically pure L-lactic acid, necessary for food applications and preferred for PLA

(poly-lactic acid, or polylactide) manufacture. The principal disadvantages of the *R. oryzae* process is the diversion of carbon away from the desired product into the byproducts ethanol and fumaric acid (see the review by Litchfield, 1996, for further details of the bacterial process, purification of lactic acid from fermentation broths, and uses of the product). Improvement in the L-lactic acid yield and product purification characteristics of the *R. oryzae* fermentation as described by Snell and Lowery (1964) consisted primarily of introducing calcium carbonate and increasing the temperature late in the production phase. These adjustments resulted in lactate yields of 72–79% and avoidance of calcium lactate crystallization during the fermentation, which simplified product purification.

From the late 1980s to the present, process optimization strategies have centered on the issue of morphology of *R. oryzae*, a universally critical parameter in fungal fermentations. These studies have taken two basic approaches, immobilization of cells (Hang *et al*., 1989; Tamada *et al*., 1992; Hamamci and Ryu, 1994; Dong *et al*., 1996; Xuemei *et al*., 1999), and promotion of mycelial or pellet morphology (Yang *et al*., 1995; Kosakai *et al*., 1997; Du *et al*., 1998; Park *et al*., 1998; Yin *et al*., 1998; Zhou *et al*., 2000). The term *pellet morphology* can be the source of some confusion in the discussion of optimal fungal morphology, since pellets of less than about one millimeter are associated with high production rates and yields, whereas larger pellets are not. Presumably, this is due to mass transfer limitations with regard to oxygen, substrates, and products. Process parameter optimization leading to consistent production of small pellets would probably be the most economical means of obtaining a high-yielding strain. A number of studies have reported yields (w/w) of 85% to 88% (Kosakai *et al*., 1997; Longacre *et al*., 1997; Yin *et al*., 1997; Du *et al*., 1998; Zhou *et al*., 1999). These yields are comparable to the yields routinely obtained with the bacterial process, and if consistently obtained, would contribute greatly to the economic competitiveness of the fungal process.

Considerable progress has been made in understanding the physiology and biochemistry of acid production by *R. oryzae*. Early studies demonstrated that *R. oryzae* produced L-lactate via glycolysis with the concomitant production of ethanol and carbon dioxide (Waksman and Foster, 1938; Gibbs and Gastel, 1953; Margulies and Vishniac, 1961). These studies also showed that lactic acid yield was increased and ethanol formation decreased under aerobic conditions, while the opposite was true under low oxygen conditions. Wright and coworkers have developed a computational metabolic model for *R. oryzae* based on elegant radioisotope studies of intracellular and extracellular metabolite pools (Wright *et al*., 1996; Longacre *et al*., 1997). This model and the accompanying radiolabeling experiments with cultures of *R. oryzae*, have provided important insights for improving the yield of lactic acid (Longacre *et al*., 1997; Wright *et al*., 1996). A simplified scheme of metabolism in *R. oryzae* shows the critical reactions in the formation of organic acids and ethanol (Figure 12.4). The biosynthesis of L-lactic acid, L-malic acid, fumaric acid, and ethanol occur in the cytosol with pyruvate at the crossroads leading to production of each compound. The addition of carbonate has the desirable effect of decreasing ethanol production, presumably through the inhibition of pyruvate decarboxylase, but the undesirable effect of increasing malate and fumarate production through the stimulation of pyruvate carboxylase (Lockwood *et al*., 1936; Waksman and Foster, 1938; Foster and Waksman, 1939). Through radiolabeling studies, this effect was quantified at four different sodium carbonate concentrations from 0 to 30 mM (Longacre *et al*., 1997). These studies showed that ethanol did not reach a minimum until after fumarate and malate

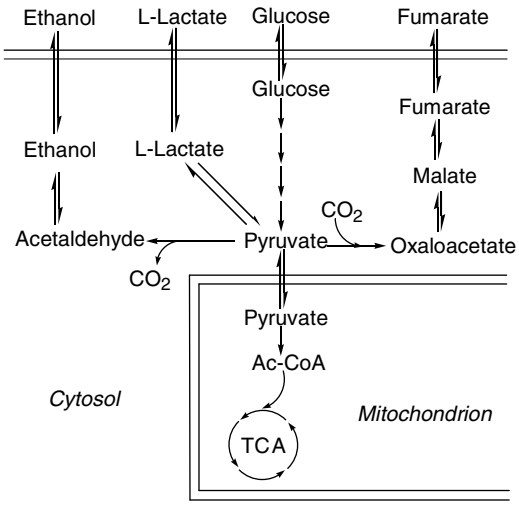

Figure 12.4. Critical pathways for organic acid synthesis in *Rhizopus oryzae*.

had begun to increase, indicating that minimizing the formation of these side products would not be possible through adjustment of carbonate concentrations alone. One strategy that has been pursued is to isolate mutants with decreased ethanol production, which is normally maximal under anaerobic conditions, so that the organism can be grown anaerobically in the production phase, which eliminates metabolic flux to malate and fumarate. Longacre *et al*. reported the isolation of such a mutant attaining 86% yield of lactic acid production. Similarly, Skory (1998) obtained a mutant with decreased alcohol dehydrogenase activity that produced relatively high concentrations of lactic acid under anaerobic conditions. However, even with the greatly decreased alcohol dehydrogenase activity levels, the mutant strain still produced substantial amounts of ethanol under anaerobic conditions. These results suggest that the complete elimination of ethanol production would be desirable and the logical genetic target would be the first committed step in ethanol biosynthesis, and the cloning of two pyruvate decarboxylase genes from *R. oryzae* suggests this strategy is already being pursued (GenBank accession numbers AF282846 and AF282847).

The enzymology and genetic control of the key step in lactate synthesis by *R. oryzae* is now well understood. *R. oryzae* possesses three L-lactate dehydrogenases (LDH), including one NAD-independent LDH (Pritchard, 1971) and two NAD-dependent LDH isozymes (Pritchard, 1973; Yu and Hang, 1991; Skory, 2000, 2001). The D-(−)−lactate dehydrogenases present in bacteria, chytridiomycetes, and oomycetes have not been reported in the phyla Zygomycota, Ascomycota, and Basidiomycota (Gleason *et al*., 1966; LéJohn, 1971; Wang and LéJohn, 1974). The cDNAs for two NAD-dependent LDH isozymes, *ldhA* (AF226154) and *ldhB* (AF226155), have been isolated and sequenced (Skory, 2000). PCR studies with gene-specific primers indicated that the two NAD-dependent LDH genes are expressed differentially; *ldhA* is expressed in the presence of glucose, xylose, or trehalose, whereas *ldhB* is expressed only on the non-fermentable carbon sources, ethanol, glycerol, and lactate (Skory, 2000). The cumulative results of this study suggested that *ldhA* encodes

an LDH biased toward the reductive reaction (pyruvate to lactate) and *ldhB* encodes an LDH biased toward the oxidative activity (lactate to pyruvate). Unequivocal demonstration of these catalytic characteristics await independent expression of the genes in a heterologous host and analysis of the pure isozymes. If *ldhB* encodes an L-lactate oxidizing isozyme then inactivation of this gene might minimize degradation of product at later stages of L-lactate production processes.

3.2.2. Fumaric Acid

In addition to producing L-lactate, *Rhizopus* spp. are also the best of the identified fungal sources for fumarate production. Ehrlich (1911) first identified fungal fumaric acid production in a strain of *Rhizopus nigricans*. A later survey of 41 strains from eight genera of Mucorales identified *Rhizopus, Mucor, Cunninghamella*, and *Circinella* spp. as producers of fumarate, though this property occurred with the greatest frequency in *Rhizopus* spp. (Foster and Waksman, 1939). The nutritional and physical requirements of *R. oryzae* leading to maximum yields of fumarate have been examined (Rhodes *et al*., 1959). Like other fungal fermentations accumulating high concentrations of organic acids, high carbohydrate concentrations, and high carbon to nitrogen ratios are conducive to high fumaric acid yields with minimal biomass accumulation. Conversion of 60% to 70% of the sugar to fumaric acid (w/w) was achieved in vigorously agitated submerged cultures containing 10–12% glucose and C : N ratios ranging from 120 : 1 to 150 : 1. Standard minerals and calcium carbonate were also added after 3–8 days, and the cultures were incubated at 33°C (Rhodes *et al*., 1959). Recently, pH and metal (magnesium, zinc, iron, and manganese) concentrations were varied with the result that consistent pellet morphology (about 1 mm pellets) and relatively high fumarate output was obtained (Zhou *et al*., 2000). Unfortunately, the strain of *R. oryzae* (ATCC 20344) used in these experiments produced high concentrations of ethanol under these conditions, leading to relatively low weight yields (39–46%) of fumarate.

The carbonate in fumaric acid production media is required to neutralize and precipitate the fumaric acid. In addition, carbonate is necessary for the formation of oxaloacetate by pyruvate carboxylase. The metabolic model developed for an L-lactate synthesizing strain of *R. oryzae* also has bearing on fumarate synthesis. The results of those modeling studies indicated that increasing carbonate concentrations raised fumarate and malate yields at the expense of lactate yields. This is consistent with the requirement for high concentrations of carbonate in fumarate production strains (Rhodes *et al*., 1959). An increase in pyruvate carboxylase activity was observed to correlate with glucose utilization and fumarate production in *R. oryzae* (Overman and Romano, 1969). Pyruvate carboxylase is located in the cytosol of *R. oryzae* (Osmani and Scrutton, 1985), as is one of two fumarate hydratase isozymes (Peleg *et al*., 1989b). A fumarate hydratase gene (*fumR*) has been cloned from *R. oryzae*, and found to encode a potential mitochondrial targeting sequence (Friedberg *et al*., 1995). The cytosolic fumarate hydratase activity increases during the production stage of the fumarate fermentation (Peleg *et al*., 1989b). However, it appears that *R. oryzae* contains a single *fumR* gene and a single mRNA transcript from this gene, so the mechanism of the observed increase in fumarate hydratase activity during fumarate production conditions remains unclear. *R. oryzae* contains both cytosolic and mitochondrial isozymes of NADP-malate dehydrogenase (decarboxylating) and NAD-malate dehydrogenase

(Osmani and Scrutton, 1985; Peleg *et al*., 1989b). The decarboxylating malate dehydrogenases have the potential to create a futile cycle between pyruvate and malate. Thus, eliminating the gene encoding the cytosolic isozyme of NADP-malate (decarboxylating) might have a beneficial effect on yield. The cytosolic location of pyruvate carboxylase, malate dehydrogenase, and fumarate hydratase indicates that fumarate synthesis can occur by a reductive pathway located exclusively in the cytosol (Osmani and Scrutton, 1985; Peleg *et al*., 1989b). However, this does not exclude a contribution from the TCA cycle (Kenealy *et al*., 1986).

Strategies to increase fumarate production include obtaining consistent and favorable morphology as discussed above (Zhou *et al*., 2000). It appears that the strain employed in the cited study would benefit from disabling the ethanol production pathway by eliminating the pyruvate decarboxylase or alcohol dehydrogenase genes. Kenealy *et al*. (1986) showed that fumarate hydratase specific activity is greater for the reverse reaction, L-malate to fumarate (at least *in vitro*), suggesting that the rate of fumarate production might be increased by removing the fumarate from the cytosol more rapidly. In analogy to the suggestion for the citric acid process, increasing the expression of the membrane transporter of fumarate (dicarboxylic acid transporter) may elicit the desired increase in rate and yield of fumarate production.

3.2.3. L-Malic Acid

L-Malic acid production has been observed in *R. oryzae* (Longacre *et al*., 1997) and in *Aspergillus* spp. (Abe *et al*., 1962; Peleg *et al*., 1988, 1989a; Bercovitz *et al*., 1990; Battat *et al*., 1991). Generally, L-malate accumulation in *R. oryzae* is minor compared to L-lactate or fumarate. Clearly, the mechanism leading to malate production in *R. oryzae* is the same as the pathway leading to fumarate, abbreviated by one step. If the cytosolic isozyme of fumarate hydratase could be decreased, the transformation of a fumarate producing strain of *R. oryzae* into an L-malate producing strain would be possible. However, the ambiguity regarding the mechanism leading to increased cytosolic fumarate hydratase activity in *R. oryzae* renders manipulation of the organism problematic.

A variety of *Aspergillus* spp. have been found to produce L-malic acid (Abe *et al*., 1962; Bercovitz *et al*., 1990). Bercovitz *et al*. (1990) tested 13 strains, representing nine species, of *Aspergillus* for L-malic acid production and found yields of 1%–4% (w/v). An *A. flavus* strain (ATCC 13697), was found to be the best producer of L-malic acid, confirming the earlier results of Abe *et al*. (1962). However, this strain had some of the lowest levels of cytosolic pyruvate carboxylase and NAD-malate dehydrogenase activities of the strains tested, suggesting that flux through this portion of the metabolic pathway is not rate-limiting (Bercovitz *et al*., 1990). Through manipulation of standard fermentation parameters (agitation, aeration, glucose, nitrogen, phosphate, and metals), an efficient process for production of L-malate was developed and yields up to 128 mole percent (95 weight percent) were reported (Battat *et al*., 1991). Interestingly, the addition of 50 ppb of Mn^{2+} led to a precipitous decline in acid production, which is consistent with the effect of this metal on citric acid production by *A. niger*. Unfortunately, as the authors note, the use of *A. flavus* has undesirable implications for the production of food grade L-malate, viz, the possibility of aflatoxin contamination. Another characteristic of this organism was the production of significant quantities of succinic acid and, to a lesser extent, fumaric acid. *Aspergillus sojae* (ATCC 46250, a soy sauce producing strain) actually appeared to be the best candidate with

regard to minimal contaminant, acid production, and food safety. Perhaps a broad survey of non-aflatoxigenic *Aspergillus* spp. would prove useful for identifying an organism producing high yield of L-malic acid and low levels of byproduct acids.

3.2.4. Succinic Acid

Most of the emphasis on biological succinic acid production has been on bacterial fermentations, as relatively efficient processes have been developed for anaerobic bacteria (see Zeikus *et al*., 1999, for a recent review). However, *Fusarium* spp. (Foster, 1949), *Aspergillus* spp. (Bercovitz *et al*., 1990), and *Penicillium simplicissimum* (Gallmetzer *et al*., 2002) are known to produce and secrete the acid. The L-malic acid producing *Aspergillus* spp. secreted succinic acid as a secondary product at lower concentrations, the highest titer cited was only 1.3%, representing 25% of the total organic acid production (Bercovitz *et al*., 1990). The formation of succinate from glucose by *P. simplicissimum* was investigated under aerobic and anaerobic conditions. This fungus secreted low levels of succinate; the highest rate was 0.063 grams succinic acid per gram dry weight mycelium per hour (final concentrations were not reported) with respiration inhibited by 5 mM sodium azide. Nevertheless, the results are interesting for three reasons: succinic acid was the predominant acid produced under anaerobic conditions, pellet formation (diameter not reported) was shown to be important in obtaining maximum succinate production rates, and it raised the possibility of fumarate respiration as a biochemical mechanism for succinate production under anaerobic conditions.

There are three possible metabolic mechanisms for production of succinate: the oxidative portion of the TCA cycle, the reductive portion of the TCA cycle, or the glyoxylate bypass (Figure 12.5). Metabolism by either the oxidative portion of the TCA cycle or the glyoxylate bypass pathway conserves only four of the six carbons from glucose in the four-carbon succinic acid product. On the other hand, the reductive portion of the TCA cycle produces two four-carbon acids for every glucose molecule metabolized via glycolysis operating in conjunction with pyruvate carboxylase. Thus, anaerobic metabolism is preferred for succinic acid producing microorganisms. Gallmetzer *et al*. (2002) suggested that succinate production in *P. simplicissimum* may occur via fumarate respiration but this has not been demonstrated. The physiology of succinate production by filamentous fungi is an emerging field but the current knowledge suggests it may be a promising field. A screening strategy might identify more promising fungal strains that combine the features of high succinate rates and final titers, low side-product concentrations, and tolerance to low pH.

3.2.5. (−)−*trans*-2,3-Epoxysuccinic Acid and *meso*-Tartaric Acid

(−)−*trans*-2,3-Epoxysuccinic acid (ESA) was first isolated from two species of fungi in 1939 (Sakaguchi *et al*., 1939). These fungi are currently designated *Paecilomyces variotii* (NRRL 1282) and *Talaromyces flavus* (*Penicillium vermiculatum*, NRRL 1009) (Martin and Foster, 1955). A third ESA producing fungus identified as an *A. fumigatus* strain (NRRL 1986, no longer available) was identified in 1945 (Birkinshaw *et al*., 1945). Optimization of fermentation parameters for *P. variotii* (NRRL 1123) on 12% (w/v) glucose, resulted in a 41% weight yield of ESA representing 61% of theoretical (one ESA molecule per glucose molecule) (Ling *et al*., 1978).

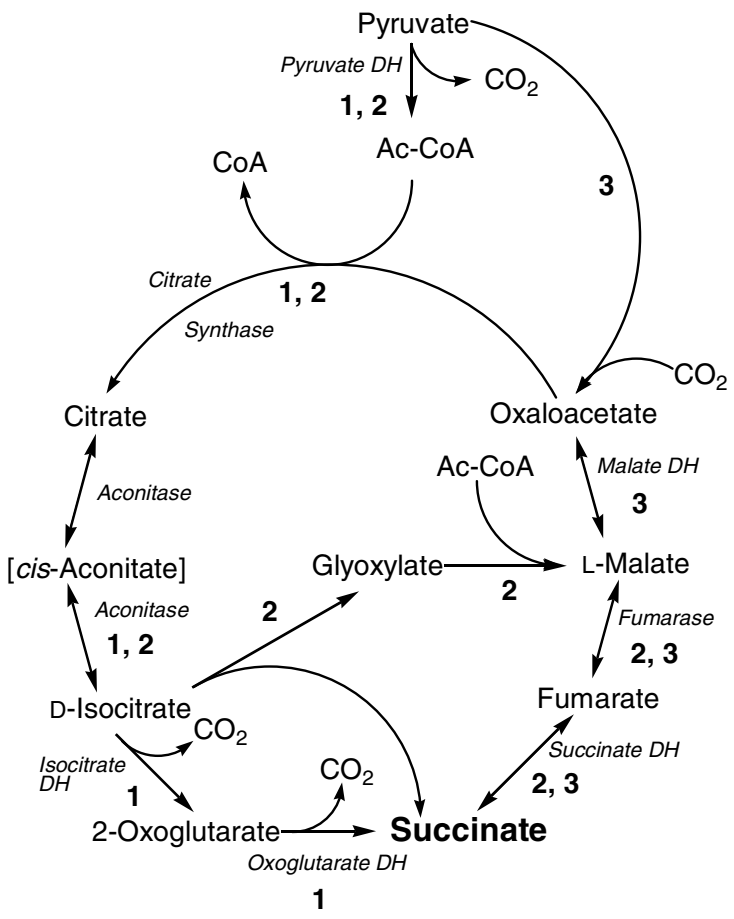

Figure 12.5. Three potential succinate biosynthetic pathways. 1. Oxidative TCA pathway. 2. Glyoxylate bypass pathway. 3. Reductive TCA pathway.

Like oxalate and itaconate biosynthesis, ESA production is only one step removed from the citric acid cycle. ESA appears to be formed directly from fumarate. Studies with $^{18}O_2$ and $H_2{}^{18}O$ showed that ^{18}O was incorporated into ESA from the former but not the latter (Aida and Foster, 1962; Wilkoff and Martin, 1963). Feeding [1,4-^{14}C]fumarate resulted in ESA labeled exclusively in the carboxyl carbons, whereas incorporation of [2,3-^{14}C] fumarate resulted in ESA labeled only in the epoxide carbons (Wilkoff and Martin, 1963). Thus, ESA appears to be derived via the addition of oxygen to the double bond in fumaric acid by an unidentified enzyme utilizing molecular oxygen (Figure 12.6). The hydration of ESA to *meso*-tartaric acid is catalyzed by fumarase from pig heart and is likely to be the general biological mechanism by which this reaction occurs (Albright and Schroepfer, 1971). Based on three-dimensional structural energy minimization *meso*-tartaric acid appears to have a rather linear structure (the carboxyl groups are "anti"), possibly suitable

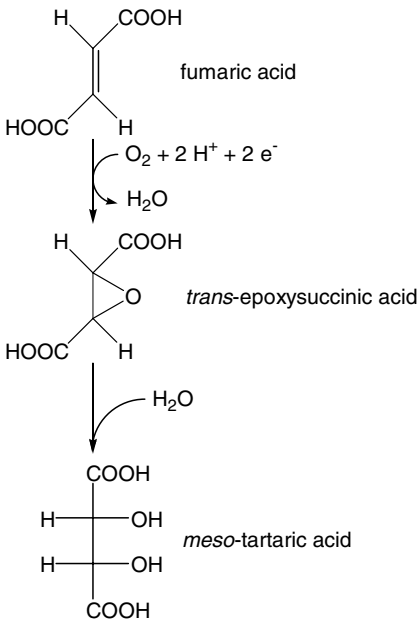

Figure 12.6. Presumed biosynthetic pathway for *trans*-epoxysuccinic acid and *meso*-tartaric acid.

for copolymer formation with diols, whereas the grape-derived tartaric acid of commerce L(+)-tartaric acid has the carboxyl groups in a "syn" conformation consistent with its use as a chelator.

4. Final Perspective

We are entering an era of accelerating development of novel fungal fermentations due to the explosion of information and tools to exploit this information. An increasing number of fungal genomes are being sequenced. The information from fungal genome sequences will accelerate and simplify the identification of metabolic pathways, the repertoire of catabolic enzymes available to an organism, the uptake and export mechanisms, and potential promoters, regulatory genes, etc. Microarray analysis and proteomics can be used to assess the expression and translation of functional gene products under different physiological (fermentation) conditions. Increasingly sophisticated models can be used to predict rational targets for metabolic engineering. Critical genetic loci implicated by traditional mutagenesis and screening programs may be functionally identified with the new information and tools available. An increasing appreciation of the diversity of fungi and the under-explored nature of this Kingdom is arising. High throughput culturing and screening tools are available to assess the potential utility of known and novel fungi. These developments in information, tools, and attitudes have the potential to accelerate the development of novel, efficient, economically feasible, and environmentally responsible fermentations.

One can now envision a fermentation development process where a desired product is chosen and the following sequence of questions is asked to identify or create a fungus with the requisite properties:

- Are there characterized fungi known to synthesize the product?
- Does a rapid screen of uncharacterized fungi reveal a producer?
- Are the biosynthetic pathways to the desired product known?
- Can a fungus with desirable fermentation behavior (e.g., wide substrate utilization range, and defined media requirements leading to simpler product purification) be genetically engineered to produce it?

Certain companies are already following this systematic approach to developing new processes and they will likely be the strongest competitors in the future.

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