C_4 Acid production in eukaryotes

Abbott 2009

Eukaryotes are better for the production of C_4 organic acids as prokaryotes often have complex nutritional requirements due to a limited ability to synthesise B-type vitamins and amino acids. Feed and downstream processing costs are increased. Prokaryotes often can't produce acids or survive at pH values where the acids are present in undissociated form, thereby increasing costs.

Natural eukaryotic producers of C_4 organic acids are a good option as they are easily obtainable once discovered. Their growth morphology is, however, dependant on their extracellular environments (pellets or biofilms), thus making them harder to control and predict. Some like Aspergillus flavus also produce aflatoxins during acid production, which presents safety and separatory challenges.

Another option regarding eukaryotes is taking a well-known, well studied, easily obtainable eukaryote that can survive and grow with simple feed media, survive at a low pH and has predictable growth and production characteristics. Saccharomyces cerevisiae is ideal for this application. Yeast does, however, not produce these acids naturally and needs to be modified. This can be done classically through random mutations and natural selection of the best producers or through genetic manipulation. Genetic manipulation is an attractive choice as results are more targeted and unambiguous, although not necessarily immediately perfect.

When modifying S. cerevisiae, a few alterations on the metabolism is necessary. Firstly, alcoholic fermentation must be eliminated as this is a major glucose sink in batch cultures. Secondly, desired product pathways must be established taking redox and energy constraints into consideration. Thirdly, products must be exported out of the cell effectively. Fourthly, the organism must be able to tolerate its substrate, products and environmental conditions.

To eliminate ethanol formation, either pyruvate decarboxylase (PDC) or alcohol dehydrogenase (ADH) must be targeted. A third option is by intervening in cellular regulation and hexose transport when in the presence of excess glucose, effectively eliminating the Crabtree effect. ADH inhibition leads to an intercellular build-up of toxic acetaldehyde. Knocking out PDC causes the organism to be sensitive to high glucose concentrations and, when using defined media, requires some C_2 source to survive. This C_2 dependence and glucose sensitivity was eliminated through natural selection. A high yield of pyruvate is achievable using this process (Y_{SP} = 0.54; c_P = 135 g/L).

The genes to import malate for consumption were imported to S. cerevisiae from a wine yeast. A combination of importing a pyruvate carboxylase overexpression, malate dehydrogenase overexpression and expression of a wine yeast malate transporter yielded better results than each individual improvement.

The ideal production scenario would be where an organic acid is produced inside the cell, exported to the outside of the cell at minimal cost and then precipitated at high enough titre.

At neutral or higher pH values, export of dicarboxylic acids have small energy costs (assuming the organism possesses the enzyme required for export of the specific acid) as there is a small or no diffusion gradient trying to push undissociated acids back into the cell.

As the concentration of dissociated acids in the fermenter broth increases, the pH decreases. As the pH decreases, a point will be reached where some of the acids start to dissociate (near the pK_a value). These undissociated acids then diffuse back into the cells, requiring more energy to actively transport them out again. This causes futile cycling as it increases the production energy costs.

If at high enough titre, the undissociated acids will reach their solubility limits in water and crystallise out and can then easily be separated downstream in the process.

Transport cost can impose an upper limit on the maximum acid yield in batch fermentation if pH is not regulated.

H^+-ATPases are the most common and abundant form of intracellular pH control in S. cerevisiae. They actively transport H^+ cations to the outside of the cell membrane. Some other regulons also supplement this effect.

Other toxification effects than decoupling can also occur, e.g. apoptosis or reaction with reactive oxygen species (ROS).

Shah 2016

Saccharomyces cerevisiae was used as it can survive at low pH values and does not form biofilms or pellets.

The operating pH was kept below the lowest pK_a of fumaric acid (3.00) to ensure at least 50% undissociated acid. After high enough titre is achieved, this acid should then precipitate out.

S. cerevisiae had to be modified to allow for fumaric acid to be exported out of the cell as transporters were a major bottleneck in the metabolism. The gene for fumerase was also knocked out.

A thermodynamic analysis was done to determine the most suitable route.

The transporter from Aspergillus niger was used. No information on the mechanism and proteins required for transport of fumerate in Rhizopus spp. Are known.