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Fermentation 2015: The ABE process

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Dr Lee Colyer Speight of Green Biologics describes an often-overlooked fermentation process for speciality chemical production

Renewable chemicals continue to grow in interest for academic researchers, policymakers and producers of industrial chemicals. Despite this, the science behind most renewable chemicals is often poorly understood, due to many processes being protected by the companies and institutions behind the technologies.

Fermentation science, however, is an ancient art and most of the details of how fermentation pathways lead to valuable industrial and speciality chemicals are well understood. Whilst most are familiar with alcoholic fermentation by yeast used to produce ethanol, another type of fermentation is remarkably similar but less well understood: acetone, n-butanol and ethanol (ABE) fermentation.

First discovered by Louis Pasteur in 1861, ABE fermentations were brought to the world's attention in 1912 when British chemist Dr Chaim Weismann invented a process for using *Clostridia* bacteria to produce high-purity acetone for the Allies' war effort. Using starch or molasses, this technology spread throughout the world until propylene-based technologies became industrially favoured in the 1950s. ABE technology ceased production in South Africa in the 1980s. Now, several biotechnology companies are championing its renaissance.[1]

Metabolically, ABE fermentation, like lactic acid fermentation in animals or alcoholic fermentation in yeast, begins with glycolysis. Glycolysis is most commonly known for its production of adenosine triphosphate (ATP), which is used as the main energy currency in cells. However the consumption of redox factors, such as nicotinamide adenine dinucleotide (NAD+/NADH,) creates a redox imbalance that must be corrected through respiration, either with oxygen or anaerobically through fermentation.[2]

ATP production begins when glucose is phosphorylated by hexokinase, actually consuming one equivalent of ATP. The product, glucose-6-phosphate, is then isomerised by phosphoglucose isomerase to fructose-6-phosphate, which is further phosphorylated by phosphofructose kinase - consuming a second equivalent of ATP - to produce fructose-1,6-diphosphate.

Up to this point, the process has consumed two equivalents of ATP. These are valuable investments, because the hydrolysis of ATP is energetically favourable, rendering the process irreversible. Additionally, the installation of two charged phosphate groups onto an otherwise uncharged sugar molecule prevents its free diffusion out of cells.

The next enzyme in the process, fructose-bisphosphate aldolase, cleaves fructose-1,6-diphosphate into two isomeric molecules, dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GADP). This transformation is a marvel for synthetic chemists, as it is a reverse aldol reaction that proceeds with complete stereochemical control to produce

isomerically pure D-GADP. Since only GADP is the substrate for the subsequent steps, an isomerase (triosephosphate isomerase) is present that interconverts the two species.

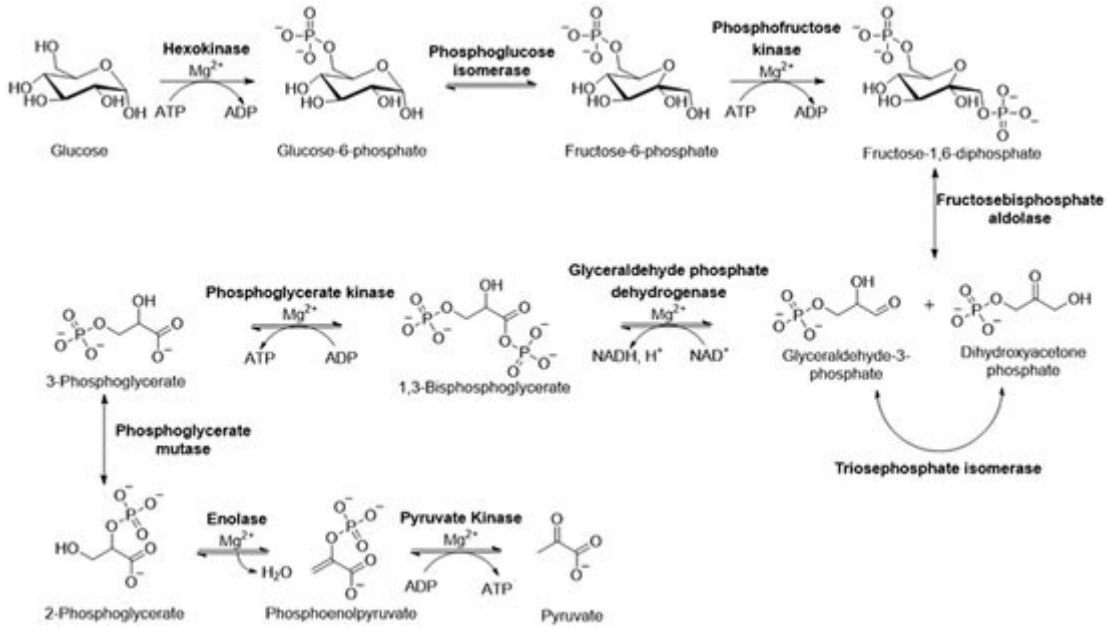

Figure 1 – Conversion of glucose into pyruvate via canonical glycolysis process

Here, two molecules of GADP have been produced from each molecule of glucose that has entered this pathway and the pay-off phase of the process can begin. GADP is then net-oxidised and phosphorylated by the consumption of NAD+ and inorganic phosphate (Pi) to produce D-1,3-bisphosphoglycerate (1,3-BPG). 1,3-BPG, containing a liable mixed phosphoanhydride, can now energetically and favourably phosphorylate adenosine diphosphate (ADP) to replace a molecule of ATP.

The enzyme phosphoglycerate kinase catalyses this production of ATP and the transformation of 1,3-BPG into 3-phosphoglyercate. As this occurs twice - one glucose molecule produces two 1,3-BPG molecules - all of the energy currency required to begin the process has been replaced, but there has been a net consumption of NAD+. Therefore, while the process is energetically balanced, a redox imbalance is beginning.

ATP production continues as S-3-phosphoglycerate is isomerised by phosphoglycerate mutase into R-2-phosphoglycerate. R-2-phosphoglycerate is then dehydrated by enolase to produce phosphoenolpyruvate (PEP). This dehydration to produce the phosphoenolate is akin to synthetic chemistry reactions to make a better leaving group, as the phosphate group is now adjacent to the alkene, allowing the terminal phosphate of ADP to attack the phosphate group on PEP in such a way that electrons can be shuffled across the oxygen and two carbons to form a transient anion that is immediately protonated. The resulting organic product is pyruvate (Pyr, Figure 1).

At this point the redox imbalance is apparent. Typically, aerobic respiration allows for the regeneration of NAD+ during the electron transport chain in which oxygen oxidises NADH back into NAD+, also producing water in the process. When there are low levels of oxygen, anaerobic respiration is necessary as oxygen is unavailable and another electron acceptor is necessary.

In animals, the electrons from NADH are transferred to Pyr to create L-lactic acid and restore an equivalent of NAD+. This lactic acid fermentation process frequently occurs during exercise and the build-up of lactic acid is responsible for muscle soreness.

Yeast, another eukaryote, is perhaps the best known fermenting organism and uses a different pathway to restore redox balance in the absence of oxygen. In yeast, Pyr is decarboxylated by pyruvate dehydrogenase to form acetaldehyde, producing two molecules of molecules of CO_2. Acetaldehyde is then reduced by NADH to ethanol (enzymatically catalysed), restoring the NAD+ equivalents necessary to conduct the production of pyruvate. In both of these examples, oxidising agents are restored in less than two steps.

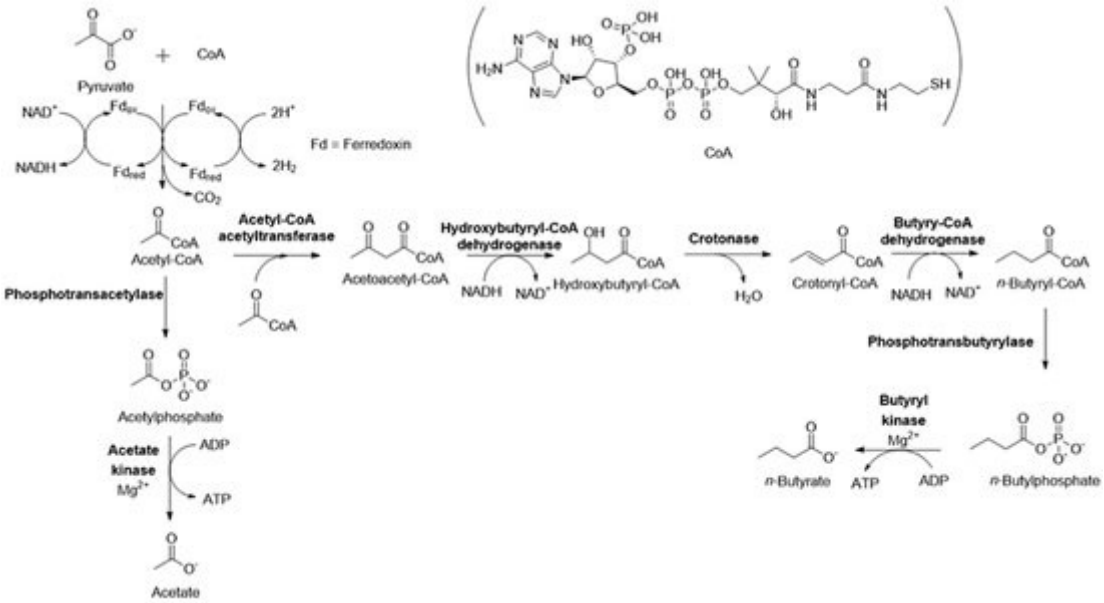

Figure 2 – Production of organic acids during acidogenesis phase of ABE fermentation & their efflux from cells

Clostridia bacteria, however, have evolved a rather unique solution to restoring the redox balance post-glycolysis under fermentative conditions. Like aerobic respiration for eukaryotes, ABE fermentation in *Clostridia* begins with the conversion of Pyr into acetyl-CoA through the decarboxylation of Pyr and subsequent transfer of the produced acetyl group to Coenzyme A (CoA).

This process consumes another equivalent of NAD+, bringing the total deficit to four per molecule of glucose consumed. Acetyl-CoA from this transformation can then have a variety of fates in the following pathways, but it is best to examine the production of acetate and n-butyrate, the intermediates of acetone, n-butanol, and ethanol, separately for the time being.

Acetate is produced from acetyl-CoA via a two-step process. First, acetyl-CoA is turned into acetylphosphate by the enzyme phosphotransacetylase and an equivalent of Pi. Acetylphosphate is then dephosphorylated by acetate kinase, producing another equivalent of ATP and acetate.

n-Butyrate production is a bit more complicated as it involves six steps, but these structural manipulations begin the restoration of NAD+ levels. First, two molecules of acetyl-CoA are used by the thiolase enzyme acetyl-CoA acetyltransferase (THL) to produce acetoacetyl-CoA and liberate a molecule of CoA.

Hydroxybutyryl-CoA dehydrogenase (HBD) catalyses the reduction of acetoacetyl-CoA into hydroxybutyryl-CoA through the oxidation of NADH into NAD+. Hydroxybutyryl-CoA is

then dehydrated by the enzyme crotonase (CRO) to from water and crotonyl-CoA, which is reduced by butyryl-CoA dehydrogenase, restoring another equivalent of NAD+ as a molecule of NADH is again used as the reducing agent. Here, the process mirrors that of acetate production as butyryl-CoA is acted upon by phosphotransbutyrylase to form butyrylphosphate by the consumption of Pi, which is subsequently used to phosphorylate an equivalent of ADP, producing another ATP molecule and n-butyrate (mediated by butyryl kinase).

The acetate and n-butyrate produced here are effluxed from the cells and accumulate in the media. This phase of ABE fermentation is called the acidogenesis phase as it is characterised by rapid growth, the efflux of acids and a subsequent drop in the pH of the media.

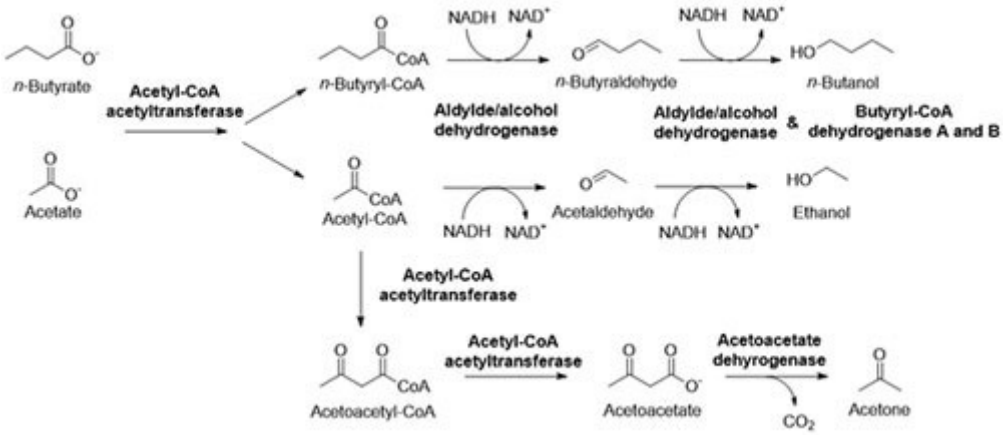

Figure 3 – Reassimilation & reduction of organic acids in solvent during solventogensis phase of ABE fermenation

This drop in extracellular pH changes the cellular physiology significantly. Both acetate and n-butyrate are taken back into the cells, which are no longer dividing at exponential rates, and reassimilated onto CoA. This influx of organic acids begins the solventogenesis phase of ABE fermentation where the titular solvents are produced (Figure 2).

Acetate and n-butyrate, once back inside the cells and attached back to CoA by acetyl-CoA transferases, are used to regenerate NAD+ and are simultaneously transformed into valuable solvents. n-Butyryl-CoA is reduced by aldehyde/alcohol dehydrogenase (AAD), producing NAD+ from NADH, to cleave the four carbon chain from CoA and concurrently form n-butyraldehyde.

n-Butyraldehyde is then reduced either by AAD or another dehydrogenase, depending on conditions and *Clostridia* strain. This final manipulation of n-butyryl-CoA transforms n-butyraldehyde into n-butanol and reforms another equivalent of NAD+.

Ethanol is produced in a similar fashion from acetyl-CoA. Acetyl-CoA, like n-butyryl-CoA, is reduced and cleaved from CoA by AAD whilst producing NAD+ from NADH. The resulting acetaldehyde follows the same fate as it would in yeast; enzyme mediated reduction by NADH to form ethanol and NAD+.

Acetone is produced in a different way. After the reassimilation of acetate to CoA, two molecules of acetyl-CoA are used by THL to form acetoacetyl-CoA, which is then hydrolysed by THL to form the free acid acetoacetate, which is then either spontaneously or enzymatically (strain- and condition-dependent) decarboxylated to form CO_2 and acetone (Figure 3).

ABE fermentation technology is a powerful tool that can be used to produce high purity acetone and n-butanol using a variety of biomass substrates as feedstocks. The technology is not without its limitations, because yields are typically around 0.35 gm solvent/gm sugar

feedstock and bacterial hosts are susceptible to infection by bacteriophages and competition with other microbes.

Several biotechnology companies have or are developing strains and/or separation technology to conquer these hurdles and make renewable acetone and n-butanol cost competitive with the petrochemical equivalents. Through these organisations and others' efforts, renewable chemicals will once again serve as valuable solvents and building blocks for the global chemicals industry.[3]

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