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Enzymes involved in the processing of starch to sugars

W. Douglas Crabb and Colin Mitchinson

The commercial processing of starch to mono- and oligosaccharides depends on the availability of three major enzymes – glucoamylase, alpha-amylase and glucose isomerase. Each of these enzymes has a unique pH and temperature optimum for use, and so unit operations of a starch plant reflect the varying operating conditions for each individual enzymatic step. This article will discuss how recent advances in molecular biology and protein engineering have allowed enzymes with improved operating parameters to be introduced to commercial applications, providing the starch processor with enhanced plant efficiency, lower operating costs and higher-quality products.

Starch from cultivated plants represents one of the most ubiquitous and accessible energy sources on the planet. Although corn (maize) is the major crop used by starch processors, other sources, such as wheat, rye and potato, are becoming more significant. The major use of processed starch is for the production of glucose, which is subsequently used to produce crystalline dextrose, dextrose syrups or high-fructose corn syrups (HFCS). Alternatively, the glucose may be fermented to produce other products such as ethanol, amino acids or organic acids. Because fructose production is the kernel of the corn wet-milling industry, this article will discuss those enzymes involved in the processing of corn to HFCS and the application of technology to improve processing options. The basic steps described below are common to all HFCS processes, and the reader should refer to Fig. 1 as each part of the process is discussed. More detailed treatment of the variety of industrial starch processes and the commercial enzymes (Fig. 2) used are available within two recent comprehensive books[1–4].

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Liquefaction

The object of starch liquefaction is to convert a concentrated suspension of purified granular starch into a solution of soluble, shorter-chain-length dextrins. The starch slurry is adjusted to approximately 30–35% dry solids and passed through a high-temperature heat exchanger (jet cooker) that instantaneously raises the temperature of the slurry to the gelatinization temperature (for corn starch, 70–90°C). As the anhydrous starch granules begin to swell irreversibly and the amylose in the granules becomes soluble and is released, the viscosity of the solution increases dramatically. To counteract this and provide a suitable substrate for subsequent saccharification, the starch must be partially hydrolysed. Over the past 30 years, almost all processors have replaced the original method (acid hydrolysis at 140°C or higher) with the addition of an alpha-amylase (AA) prior to jet cooking, thus improving the process and the product. AA (α-1,4-glucan-4-glucanohydrolase; E.C.3.2.1.1) hydrolyses internal α-1,4-glycosidic linkages in starch, essentially at random, to give shorter maltodextrins. In order to assure the removal of all lipid–amylose complexes, the preferred temperature of gelatinization is above 100°C. This has driven the replacement of the AA from

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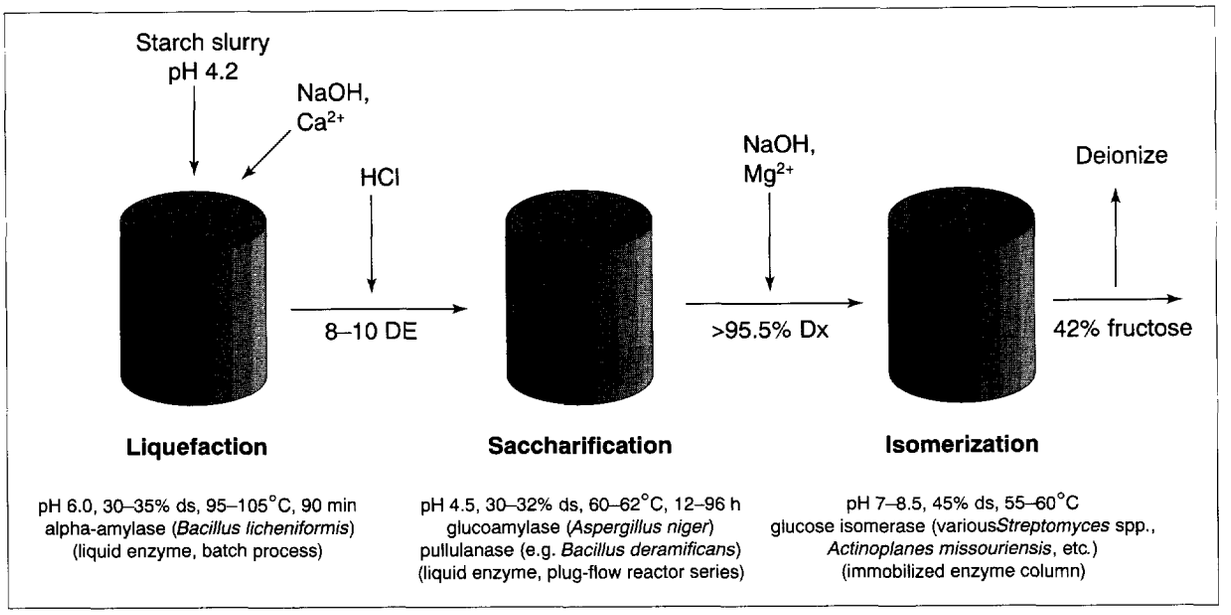

Figure 1

The starch process for high-fructose corn syrup. Schematic outline of the enzymatic steps in the processing of slurried corn starch to fructose, showing individual enzyme-usage conditions and typical processing parameters. Arrows indicate adjustment points within the process for pH and/or ion components. The process parameters may be different when producing ethanol from corn. The term 'ds' refers to the percentage of starch or glucose dry solids suspended in the slurry. DE is 'dextrose equivalent', a measure of the number of reducing ends present in a starch hydrolysate; each reducing end of an oligosaccharide is equivalent to a single dextrose residue. The greater the degree of starch liquefaction or hydrolysis, the higher the DE. Undegraded starch has a DE approaching zero; a fully hydrolysed starch would have a DE of 100. DE is related to average chain length of the oligosaccharide by the following formula: $DE = 180/(162n + 18) \times 100$, where n is the average oligosaccharide chain length. For example, a starch slurry with a DE of eight has an average chain length of ten glucose residues. The term '%Dx' is the percent of dextrose in the solution. In the example shown, after saccharification, the process stream would have 32% dry solids with greater than 95.5% dextrose (DX).

Bacillus amyloliquefaciens with the more thermostable enzymes from *Bacillus stearothermophilus* and *Bacillus licheniformis*. Although these enzymes are able to operate briefly at temperatures as high as 105°C, the process cannot be performed much below pH 5.9 as AA thermostability decreases at lower pHs. To be compatible with the pH optima of these preferred liquefaction enzymes, the pH of the starch slurry is adjusted from its natural pH of 4.5 to pH 5.8–6.5, and Ca^{2+} is added to improve enzyme stability. The next process step (saccharification) requires pH adjustment back down to pH 4.2–4.5. These adjustments increase the chemical costs and require additional ion-exchange refining of the final product for salt removal.

An AA able to operate at lower pH would reduce these costs, simplify the process and reduce high-pH byproduct (e.g. maltulose) formation in the liquefact. Amylases from extremophiles, especially thermoacidophiles, have promising properties[5–11], but none have been produced at commercially viable levels. One approach to liquefaction at lower pH, solving this problem, used a mixture of the enzymes from *B. stearothermophilus* and *B. licheniformis*[12]; another, protein engineering of the *B. licheniformis* AA, resulted in an enzyme able to operate at lower pH, lower calcium levels or higher temperatures[13]. Figure 3a shows the relative abilities of engineered and wild-type *B. licheniformis* AA to cause starch depolymerization at low pH. This same variant enzyme was also shown to be more effective than the native amylase at high temperature (107°C) across a range of Ca^{2+} concentrations (Fig. 3b) or over a range of temperatures (Fig. 3c). Thus, the engineered amylase is a generally more robust enzyme that increases the range of viable plant-operating parameters. Recently, the crystal structure of *B. licheniformis* AA has been published[14].

After jetting, the starch mixture is held at 95–100°C for up to three hours to allow the enzyme to hydrolyse the starch to an average chain length of 8–12 glucose units, thus producing the preferred substrate for saccharification.

Saccharification

The process of removing single glucose residues from a soluble oligosaccharide is termed saccharification and, in its simplest form, is catalysed by an exo-acting 1–4-glucanohydrolase (glucoamylase; E.C.3.2.1.3; GA), which sequentially removes a glucose unit from the nonreducing end until all of the oligosaccharide is degraded to glucose. The enzyme of choice is isolated from *Aspergillus niger* or a closely related species, for example *A. awamori*. *A. niger* GA has a pH optimum near 4.2, is extremely stable at 60°C, and is produced at high levels by industrial fermentation processes.

For saccharification, the pH of the liquefact is adjusted down to 4.2–4.5 and the dry solids are diluted to 30–32%. This pH adjustment serves two functions:

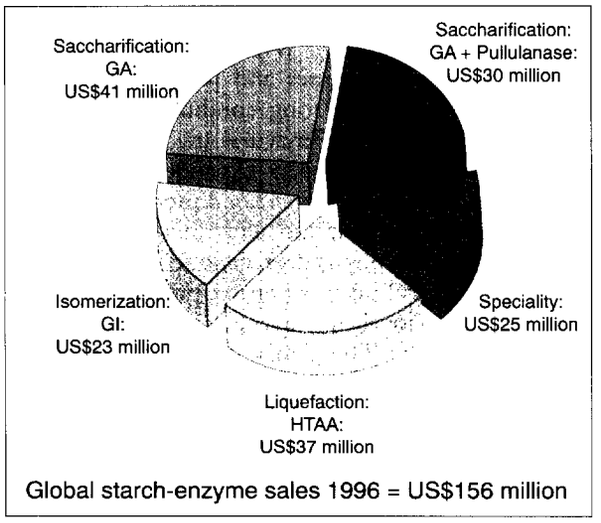

Figure 2

The global market for starch enzymes in US$. Enzymes used in the processing of starch and their relative value in the market. Speciality enzymes include acid fungal alpha-amylases, low-temperature bacterial alpha-amylases, beta-amylases and transferases. GA, glucoamylase; GI, glucose isomerase; HTAA, thermostable alpha-amylase. The GA market includes both straight GA for ethanol production and blends of GA with pullulanase for high-fructose corn syrup, and these are indicated separately.

first, it stops further action of the AA so that the liquefact maintains an average chain length optimal for saccharification; second, it moves the pH closer to the optimum for the GA. The saccharification reaction is most often performed in a series of plug-flow reactors, with the enzyme dosed so that peak dextrose is achieved between 24 and 96 hours at 60°C, depending upon plant capacity needs.

There are some practical difficulties in this process; for example, in natural starch sources, the substrate is a mixture of both amylose (1–4 linkages) and amylopectin (branched 1–6 links). Glucoamylase is efficient at cleaving the 1–4 links, but when it reaches a 1–6 branch point, the enzyme is slower to hydrolyse the bond and the result is a build-up of isomaltose. One solution to this problem has been the use of a blend of enzymes containing both GA and pullulanase (E.C.3.2.1.41), which has the ability to break the 1–6 linkages. The key to success was finding pullulanase enzymes with the same pH and temperature optima as the GA. In general, the use of the blended products increases the glucose yield from 94% to over 95.5%.

A second practical problem is that the process conditions require high dry-solids concentrations (32%) to be economical, which results in high concentrations of glucose (>95%). Under these product-enriched conditions, the GA will tend to form reversion products. In the case of 1–4 reversion products, such as maltose, an equilibrium will exist at approximately 1–2%, but 1–6 reversion products will tend to accumulate in the reaction at the expense of glucose. The solution is to balance the dosage of the enzyme, the temperature of the reaction and the reaction time so that, at later

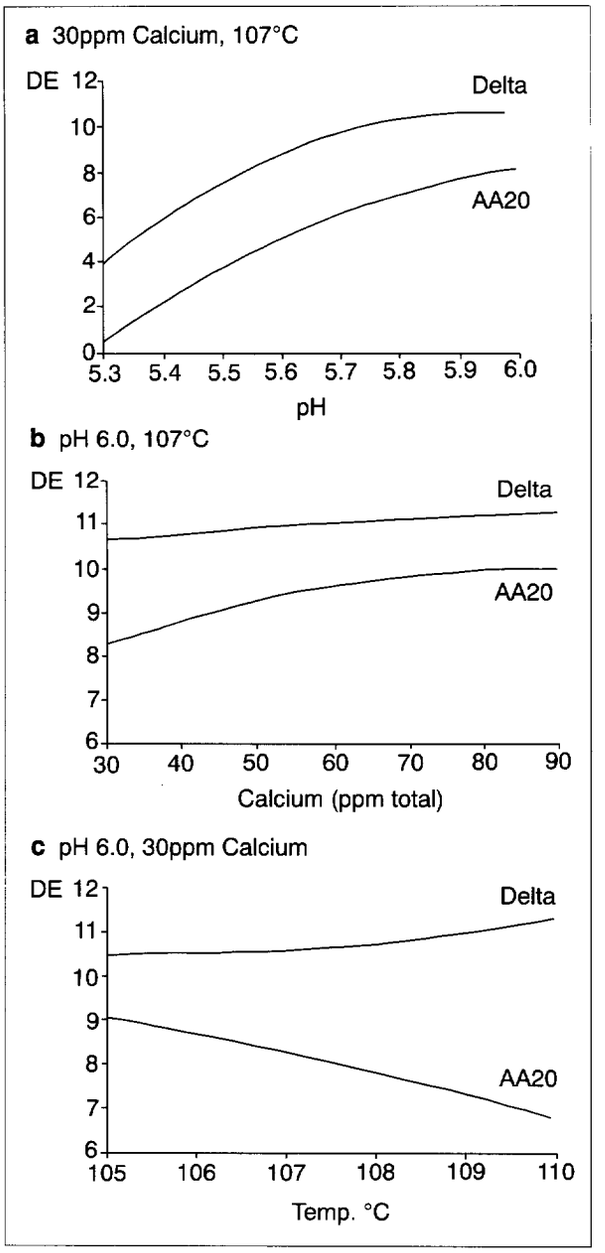

Figure 3

Improved starch liquefaction with an alpha-amylase variant. AA20 is Spezyme® AA20, a *Bacillus licheniformis* wild-type product. Delta is Spezyme® Delta AA, a *B. licheniformis* variant product. For liquefaction, a 35% dry solids wet-milled corn-starch slurry was adjusted to the indicated pH and Ca^{2+} levels. Enzyme samples were produced by fermentation of a *B. licheniformis* host containing either the native or variant amylase gene. Equal activities of the variant or native amylase were added to the slurry prior to jet cooking at the described temperatures. The dextrose equivalent (a measure of the number of reducing ends in the solution) was determined by measuring reducing-sugar content of the slurry following a 90 min hold at 95°C.

stages, thermal inactivation of the GA results in little active enzyme remaining, leading to less reversion.

One future goal in saccharification is improved thermostability of GA[15], to allow reactors to be run at higher temperatures and dry-solids levels for shorter time periods. Engineering a GA that has a decreased ability to hydrolyse (and therefore revert) 1–6 linkages could

prove highly effective in combination with improved pullulanase products (P. Coutinho, PhD Thesis, Iowa State University, USA, 1996). The crystal structure of *A. niger* GA has been recently determined[16] and the catalytic and starch-binding domains identified[17,18].

Isomerization

Following saccharification, the dextrose mixture is refined, carbon filtered, concentrated to >40% dry solids and the pH adjusted to a more neutral level (pH 7–8). An enzymatic step isomerizes the sugar aldehyde (D-glucose) to the keto form (D-fructose) by passing a concentrated glucose syrup over an immobilized column that has active glucose isomerase (D-xylose-ketol isomerase; E.C.5.3.1.5; GI) attached to a solid support. (NB Although the preferred substrate of this enzyme is xylose, the name glucose isomerase is more commonly used.) The use of an immobilized enzyme allows a continuous process and avoids the introduction of the enzyme into the product. In some cases, the support is as simple as GI-producing cells crosslinked to each other with gluteraldehyde. In other cases, the immobilized column consists of GI that has been partially purified and attached to a cellulosic resin, or of crosslinked GI crystals. The flow rate across the column is adjusted to give a retention time that results in a product stream consisting of 42–45% fructose. Several enzymes are in commercial use and, regardless of the source of the enzyme, the methodology is similar. The typical reaction conditions are pH 7–8.5 and temperatures of 55–65°C, with a fructose solution of 55% being the preferred commercial product. The thermodynamics of the operating conditions only allow a fructose solution of approximately 42% to be reached; in order to produce the 55% HFCS, a portion of the fructose stream is fractionated to yield 90% fructose syrup, which is then blended back to make a 55% fructose final product. A detailed review of the industrial GI process and enzyme technology has recently been published[19].

Direct conversion of the glucose syrup to 55% fructose would simplify the process. Theoretical calculations show that isomerizing at elevated temperatures drives the equilibrium towards fructose, and experimental data support this. However, to convert glucose directly to fructose at a final fructose concentration of 55%, the reaction temperature must approach 110°C (Ref. 20). While current enzymes may be able to survive this for short periods of time, in order to be an economic process, it would be necessary to maintain this running temperature for extended periods and, to date, no enzymes with the requisite stability have been identified. However, improved thermostable GIs have been engineered from native enzymes[21,22] or isolated from natural sources[23–25]. This technology may prove adaptable to industrial processes in the future.

Conclusions

The starch-processing industry is unique within the industrial enzyme sector, in that the use of enzymes is essential for the industry. While this has resulted in greatly improved processes and products, a consequence is that the operating conditions are limited by the properties of the enzymes used. The use parameters have already been expanded by classical approaches, for example: increased production yields that result in lower costs and allow increased dosages; removal by either physical or genetic means of side activities that can lead to reversion products; and the introduction of novel enzymes such as pullulanase and thermostable AA. The demand for enzymes with improved properties and expanded use parameters to allow simpler and more robust processes persists. These future improvements will rely on accessing nature's diversity through natural isolates or protein engineering.

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