13
Enzymes for Technical Applications

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1 Introduction .................................................................................. 380
2 Historical Outline ......................................................................... 380
3 Enzymes for the Detergent Industry .............................................. 381
  3.1 Introduction ................................................................................... 381
  3.2 History ......................................................................................... 382
  3.3 Overview of Enzymes ................................................................. 382
      3.3.1 Proteases ................................................................................ 382
      3.3.2 Amylases ............................................................................. 384
      3.3.3 Lipases .................................................................................. 384
      3.3.4 Cellulases ............................................................................. 385
3.4 Latest Innovations ................................... 386
3.4.1 Mannanases ...................................... 386
3.5 Future Perspectives .................................. 387
3.5.1 Oxidoreductases .................................... 388
3.5.2 Fabric-care Enzymes ................................. 388

4 Enzymes for the Starch Industry .................................. 388
4.1 Introduction ...................................... 388
4.2 History ......................................... 390
4.3 Enzymes ........................................ 391
4.3.1 Bacterial α-amylases ................................. 391
4.3.2 Fungal α-amylases .................................. 391
4.3.3 Glucoamylase ..................................... 391
4.3.4 Pullulanase ....................................... 391
4.3.5 β-amylase ........................................ 391
4.3.6 Glucose Isomerase .................................. 392
4.4 Latest Innovations ................................... 392
4.5 Perspectives ...................................... 393

5 Enzymes for Biofuel .................................. 393
5.1 Introduction ...................................... 393
5.2 History ......................................... 395
5.3 Enzymes and Latest Innovations ...................... 396
5.3.1 Ethanol Production Using Starch-based Raw Materials . 396
5.3.2 Fermentation ..................................... 398
5.3.3 Energy Reduction ................................... 399
5.4 Future Perspectives .................................. 399
5.4.1 Ethanol Production Using Lignocellulose-based Raw Materials . 400

6 Enzymes for the Textile Industry .................................. 400
6.1 Introduction ...................................... 400
6.2 History ......................................... 401
6.3 Enzymes ........................................ 401
6.4 Latest Innovations ................................... 406

7 Enzymes for the Pulp and Paper Industry ..................... 406
7.1 Introduction ...................................... 406
7.2 Overview of Selected Applications ...................... 407
7.3 Enzymes ........................................ 407
7.3.1 Xylanases for Bleach Boosting of Kraft Pulp . . 407
7.3.2 Cellulases and Other Enzymes for Refining of Pulp . . 407
7.3.3 Cellulase, Amylases and Lipases for Deinking and Repulping of Waste Paper 409
7.3.4 Lipases for Pitch Control . . 410
7.3.5 Esterases for Stickies Control . . 410
7.3.6 Amylases for Starch-coating Applications . . 411
7.3.7 Cellulases for Fiber Modification ........................................ 411
7.3.8 Proteases, Lipases, and Amylases for Cleaning Applications .......... 411
7.4 Future Perspectives ..................................................... 411

8 Enzymes for Organic Synthesis ........................................... 412
8.1 Introduction ............................................................. 412
8.2 History ................................................................. 413
8.3 Enzymes ............................................................... 413
8.4 Latest Innovations ..................................................... 414
8.5 Perspectives ............................................................ 414

9 Enzymes for Processing of Fats and Oils ................................ 414
9.1 Introduction ............................................................. 415
9.2 History ................................................................. 415
9.3 Enzymes ............................................................... 415
9.4 Latest Innovations ..................................................... 416
9.5 Perspectives ............................................................ 416

10 Key Technologies for the Discovery of Industrial Enzymes .......... 416
10.1 Exploring Nature’s Diversity .......................................... 417
10.1.1 Evolutionary Diversification as Basis for Enzyme Screening .... 417
10.1.2 Traditional Enzyme Screening ..................................... 417
10.1.3 Bioinformatics as a Basis for Enzyme Discovery .............. 419
10.1.4 Metagenomics or Cloning from Non-cultivable Microorganisms .. 420
10.2 Protein Optimization .................................................. 421
10.2.1 Rational Protein Engineering ...................................... 421
10.2.2 Directed Molecular Evolution .................................... 423
10.2.3 Protein Optimization Outlook .................................... 426
10.3 Conclusion ............................................................ 427

11 References ............................................................... 428

6-APA 6-aminopenicillin acid
CBD  cellulose binding domain
CBH  cellobiohydrolase
CGTase  cyclodextrin glycosyltransferase
CMC  carboxymethyl cellulose
EPA  Environmental Protection Agency
ETBE  ethyl tertiary butyl ether
HFCS  high-fructose corn syrup
L-DOPA  L-dioxyphenylalanine
MOW  mixed office waste
MTBE  methyl tertiary butyl ether
NMR  nuclear magnetic resonance
NREL  National Renewable Energy Laboratory
1 Introduction

Enzymes are major contributors to clean industrial products and processes (Bull et al., 1999). They show a variety of advantages over chemicals, e.g., their specificity, their high efficiency and their compatibility with the environment. Enzymes can be produced from renewable resources and are in turn degraded by microbes in nature. Various industries have replaced old processes using chemicals that cause detrimental effects on the environment and equipment with new processes that use biodegradable enzymes under less corrosive conditions.

Currently, industrial enzymes are manufactured by three major suppliers, Novozymes A/S (headquartered in Denmark), Genencor International Inc. (headquartered in the U.S.), and DSM N.V (headquartered in the Netherlands). Their main market segments are food (e.g., dairy, baking, brewing, beverage), animal feed, and technical applications. Novozymes A/S is the largest supplier in each of these three sectors, with an estimated market share between 41% and 44% of the industrial enzyme market in 1999. Genencor International Inc., which operates in the technical and feed segments, and DSM N.V, which focuses on food and feed, had, according to estimates by Novozymes A/S, market shares of around 21% and 8%, respectively, that year. The rest of the market is divided among a few smaller enzyme producers, some of which produce enzymes for their own use, in the U.S., Canada, Europe, and Japan, as well as a number of small local producers in China.

The applications in which enzymes are used are many and diverse. So far, technical enzymes represent the largest part of the market, with a value of approximately 1 billion USD in 1999. Enzymes for detergent are the largest single market for enzymes, with a value of around USD 0.5 billion. The other dominating markets are baking, beverage, and dairy; as well as feed and pulp and paper applications. All of these industries are traditional users of enzymes. Overall, the estimated value of the worldwide use of industrial enzymes has grown from 1 billion USD (Godfrey and West, 1996) in 1995 to 1.5 billion USD in 2000 (McCoy, 2000a).

In the following sections, we review enzymes for technical applications and briefly describe key technologies for discovery and optimization of industrial enzymes.

2 Historical Outline

Enzymes have been exploited by humans for centuries. Classical foods and beverages like cheese, yogurt and kefir, bread, beer, vinegar, wine, and other fermented drinks, as well as paper and textiles, were produced with the help of enzymes as early as 6000 B.C. in China, Sumer, and Egypt. The epoch of classical biotechnology was marked by the landmark discoveries of Leeuwenhook, who observed and described microbes; Pasteur,
who defined fermentation as a biological process; Buchner, who discovered that enzymes are proteins that function as catalysts; and Sumner, who crystallized the first enzyme. The modern era of industrial enzymology began in 1913 when Otto Röhms granted a patent for the use of a crude protease mixture isolated from pancreases in laundry detergents. In the following years, an increasing number of enzymes were found in microorganisms, and these microbes were cultured in large-scale fermentations to produce enzymes. However, the number of enzymes that could be produced in this fashion was limited, because not all microbes are amenable to large-scale fermentation. The pioneering work of Avery and MacLeod, Hershey and Chase, Watson and Crick, Cohen and Boyer, and many others who introduced the era of recombinant biotechnology revolutionized industrial enzyme production. With the advent of genetic engineering, genes encoding interesting enzymes could be transferred to and expressed in selected host microbes for industrial-scale production. Today, gene technology plays a major role in both the discovery of novel enzymes and the optimization of existing proteins and is the basis for production of the majority of industrial enzymes.

3 Enzymes for the Detergent Industry

The use of enzymes as performance enhancers in detergents is arguably the biggest innovation of the detergent industry during the past 20 years. Enzymes have found broad-based application in a variety of commercially relevant areas.

3.1 Introduction

One of the most important and profitable applications for enzymes is in detergents, where the total global market size was ~ 0.6 billion USD in 2000 (Novozymes data). The breadth of commercial detergent products is quite large, and they are used in such diverse applications as laundering, dishwashing, and in industrial and institutional cleaning (Eriksen, 1996; Olsen and Falholt, 1998). There, they have shown great utility in providing noticeable improvements in the appearance of a particular item of value to the consumer, such as clothes or dishes. Penetration of enzymes into world markets is high, with nearly complete penetration into North American, European, and Japanese markets and somewhat lower, but increasing, penetration into developing markets such as China and Latin America (Showell, 1999). The main enzyme manufacturers are Novozymes A/S, headquartered in Denmark, and Genencor International, headquartered in the U.S.

To provide desirable benefits, enzymes must be stable and function well in the presence of a variety of potentially "enzyme unfriendly" detergent ingredients (e.g., anionic/nonionic/cationic surfactants, chelants, builders, polymers, bleaches) and in various forms of detergent products (i.e., liquids and powders). In addition, many detergent manufacturers, to simplify production processes and to lower costs, have been working toward producing "globally applicable" detergents from which a single enzyme would be expected to provide its benefits across a range of global wash conditions (i.e., water hardness, presence of transition metals, chlorine) and wash practices (i.e., type of washing machine, wash temperature).

It seems clear from the growth of the detergent enzyme business that enzymes
have been found or engineered to meet these challenges. In many parts of the world, particularly in Europe and in North America, the environmental impact of detergents has been disputed (Ho Tan Tai and Rataj, 2001). This concern has influenced many detergent makers to improve the environmental compatibility of their detergents. In this vein, enzymes can be considered “ideal ingredients” in detergents of the future, since their catalytic nature allows them to provide good benefits at much lower dosage levels compared to conventional detergent ingredients such as surfactants, bleaches, and polymers. In addition, enzymes are completely biodegradable and therefore do not accumulate in the environment.

3.2 History

The first use of enzymes in detergents occurred in 1913 when Rohm & Haas introduced crude trypsin into their detergent Burnus® based on a German patent issued to Otto Röhm (1913). Issues with the performance and stability of this enzyme in their detergent, which was designed for use in a laundry presoak context to remove biological stains such as blood, did not excite consumers at the time about the potential of enzymes. It was not until Novo Industri A/S in Denmark introduced the protease Alcalase® in 1963, together with small detergent producers in Switzerland and the Netherlands, that the benefits of using enzymes in detergents became noticed. Until the 1980s, proteases were considered to be the only commercially relevant enzymes. Amylases, lipases, and cellulases were then developed, and the market began to grow substantially.

Today, many laundry-detergent products contain at least a protease, and many contain cocktails of enzymes including proteases, amylases, cellulases, and lipases. With new innovations in the areas of fermentation-process optimization and genetic modification of host organisms continually being made, as well as an increased emphasis on the environmental impact of detergents, the enzyme market will likely continue to grow in coming years.

3.3 Overview of Enzymes

Essentially all of the enzymes found in today’s detergents are hydrolytic in that they catalyze the hydrolysis of chemical bonds present within a polymeric substrate. Most commonly, these enzymes act in an “endo-” manner, meaning that the hydrolysis occurs randomly in the interior of the polymer. General descriptions of the commonly used hydrolases such as proteases, amylases, lipases, and cellulases are given below. Because of space considerations, mention is not made of methods for assessing the activity and performance of these enzymes. Discussions of these items can be found elsewhere (Eriksen, 1996; Showell, 1999; Olsen and Folholt, 1998). As discussed in the “Latest Innovations” section, mannanases, which were introduced into the detergent market in 2000 based on a collaboration between Procter & Gamble and Novozymes, have been shown to provide exciting new types of claimable benefits such as the prevention of “reappearing stains” during washing. Finally, in the “Future Perspectives” section, oxidoreductases for stain bleaching and approaches for enzymatically generated fabric-care benefits are discussed as new and exciting frontiers for detergent enzymes.

3.3.1 Proteases

Proteases are hydrolases that catalyze the hydrolysis of amide bonds within protei-
ceous substrates that are present in soils. Stains such as blood, grass, spinach, and keratin from collar and cuff soil are most relevant for laundry applications, whereas baked-on egg soils are of interest for dishwashing applications. Proteases also are used for cleaning membranes and endoscopes in the industrial and institutional area (Eriksen, 1996). These enzymes are by far the most commonly used types in detergents. Hydrolysis breaks the proteinaceous substrates down into smaller fragments (i.e., amino acids or oligopeptides), thereby increasing the ease with which the soils can be solubilized in the wash liquor by surfactants and the like. Proteases also help to prevent the redeposition of proteins on fabrics, particularly hydrophobic ones present in soils, such as blood, thereby also providing a whiteness benefit (Venegas, 1997). Figure 1 illustrates an example of the whiteness benefit that can be provided by proteases, as visualized through the redeposition of particulate soil following initial washing in detergent both with and without protease.

Nearly all commercial proteases are so-called serine proteases originating from the **Bacillus** family and contain the catalytic triad of amino acids (i.e., aspartic acid – histidine – serine) in their active sites. They are also unspecific endoproteases, meaning that they cleave peptide bonds within proteins in an unspecific way, leading to complex reaction product mixtures of oligopeptides. Given these similarities, commercial detergent proteases mainly differ in their temperature and pH optima, bleach sensitivity, and dependence on Ca and Mg ion concentration for stability. Tuning of these parameters through structural changes in the enzymes has resulted in a range of proteases that are suited to different types of tasks, i.e., improved stability in bleach-containing granulated detergents or egg-stain removal in an auto-dishwashing detergent.

Given that the function of proteases is to break down proteins, autoproteolysis and compatibility with other detergent enzymes are of concern during the formulation and storage of detergents, particularly those in the liquid form. This issue has been addressed by adding reversible protease inhibitors such as boric acid and propylene glycol to the detergent (Showell, 1999). Upon dilution of the detergent in the wash, the

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**Blood spots, after three wash cycles and re-soiling**

![Images of blood spots taken after washing in detergent with and without Savinase® and after additional particulate soil treatment.](image)

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Fig. 1  Images of blood spots taken after washing in detergent with and without Savinase® and after additional particulate soil treatment.
inhibitors are released from the enzyme active site, thereby allowing the enzyme to perform its function.

3.3.2 Amylases
Amylases are also hydrolases that catalyze the hydrolysis of glucosidic linkages in gelatinized starch polymers. Starch polymers are commonly found in foods such as pasta, fruit, chocolate, baby food, barbeque sauce, and gravy. As colored stains, their removal is of interest in both detergent and dishwashing contexts. Removal of starch from surfaces is also important in providing a whiteness benefit, since it is known that starch can be an attractant for many types of particulate soils (Ryom and Gibson, 2001). The most common class of detergent amylases is the α-amylases, which hydrolyze the 1,4-α-glucosidic bonds in starch. On some food stains, such as those from cocoa and barbeque sauce, which contain both starch and proteinaceous soils, synergistic benefits can be seen between amylases and proteases (Showell, 1999; Gormsen, 1997). Most commercial amylases derive from either the Bacillus or Aspergillus genera and typically consist of three different domains, with the active site existing between two of the domains.

3.3.3 Lipases
Lipases are a fairly new addition to the commercial detergent market. The first commercially successful lipase was introduced by Novo Nordisk A/S in 1988 under the trade name of Lipolase®, which originated from the fungus Humicola lanuginosa. Lipases break down triglycerides into their component glycerol and fatty acid units, thereby increasing their water solubility, particularly at a pH > 8 (Aaslyng et al., 1991). Owing to their hydrophobicity, these stains are among the most difficult to remove via the conventional surfactant technology commonly found in detergents (Showell, 1999). As a result, stain-removal benefits are observed on greasy/fatty stains such as lard, butter, and lipstick and on body soils such as sebum. Enzymatic removal of these soils also can generate a whiteness benefit on hydrophobic fabrics such as polyester and polyester/cotton blends, which tend to bind strongly to the unhydrolyzed soils. On cotton, oily soils can also penetrate into the lumen, and lipases can greatly aid in the removal of these soils as well (Obendorf et al., 2001).

In addition to their tenacity as stains, greasy/oily stains can, over time, undergo oxidation to form unsaturated compounds, which can impart a rancid odor and a yellowish color to fabrics (Showell, 1999). Lipases can help to remove these soils and improve the odor and appearance of fabrics.

Binding to the water-substrate interface is critical for lipase action. As one might envision, other surface-active detergent components such as surfactants can show an inhibitory effect on lipases through a blocking mechanism (Svendsen, 1997). This problem can be reduced through judicious choice of a surfactant system that contains the optimal ratio of anionic to nonionic surfactants.

Traditionally, lipase benefits have not been observed until after multiple wash cycles, and a drying step was required to show activity (Eriksen, 1996). Recent work has shown that lipases can be developed that show good benefits in the first wash cycle, as shown in Figure 2 (Callisen and Damhus, 2000). When using these lipases, a high degree of soil removal is obtained in the first wash cycle. This removal can facilitate the removal of other soil substances from the fabric by surfactants, enzymes, etc., thereby improving the overall fabric appearance.
3.3.4 Cellulases

The use of cellulases in detergents began in the late 1980s when Kao introduced an alkaline cellulase into their Attack® detergent. In contrast to the enzyme classes discussed above, the main function of cellulases in detergents is to hydrolyze the cellulose in cotton and polycotton fabrics to provide cleaning and fabric-care benefits. Cleaning effects arise from the ability of cellulases to remove particulate and oily soils. In contrast, fabric-care effects arise from the ability of cellulases to provide anti-pilling, softness, whiteness, and color clarification benefits (Gormsen, 1997). Cellulases can be either endo- or exo- and function by catalyzing the cleavage of $\beta$-1,4-glycosidic bonds in cellulose. Commercial cellulases come from both bacterial and fungal sources. They are composed of up to three different domains, including the catalytic domain, the linker, which is usually a short peptide, and a cellulose-binding domain (CBD). The CBD typically contains a hydrophobic region that affords cellulase affinity to cotton. This binding is critical for observing fabric-care benefits from these enzymes (Klyosov, 1990). As with lipases, the enzymatic mechanism requires the enzyme to first bind to the cellulose surface, followed by glycosidic bond hydrolysis.

To observe fabric-care benefits from cellulases, washing tests typically consist of multiple washing and drying cycles, since fabric decolorization and pilling increase as the garments are mechanically worn. Typically, multi-cycle tests consisting of 10 or more wash/dry cycles are needed to observe benefits, as illustrated in Figure 3.

Since cellulases act to degrade cellulose, the dosage of enzymes in the detergent must be chosen carefully to allow for fabric-care benefits to be observed without causing structural damage to the garments over their lifetime. To address this, tensile-strength-loss tests are often conducted on sensitive fabrics as a check for fabric damage. In addition to modifying dosage, tensile strength loss can be minimized by choosing a cellulase that preferentially degrades amorphous versus crystalline cel-
lulose (Lenting and Warmoeskerken, 2001). Weak, surface-based fibers are thought to consist of amorphous cellulose, and their presence is thought to give rise to much of the decolorization and pilling observed on worn cotton. In contrast, crystalline cellulose resides in the interior of the cotton, and damage to it is thought to be largely responsible for tensile strength losses.

3.4 Latest Innovations

Sometimes, the true benefit of a new detergent enzyme to the consumer may not be apparent during the early stages of its development, possibly because observation of the benefit relies on a complex set of factors that are sometimes not part of standard laboratory wash-testing protocols. This “true” benefit may be realized only after testing the effect of the new enzyme in consumers’ homes as part of a consumer test. The example below illustrates such an enzyme and shows how capitalizing on such performance benefits can lead to marketplace success.

3.4.1 Mannanases

A particularly exciting new development in the area of detergent enzymes was the introduction of Mannaway® in Procter & Gamble’s Tide Deep Clean® liquid detergent formula in 2000. This enzyme, which was developed in collaboration with Novozymes, addresses a key cleaning problem commonly seen in consumers’ laundry. This problem revolves around the tendency of certain polysaccharides such as guar gum and starch to strongly bind particulate soils. Guar gum is used commonly as a thickener in foods, and its cationic variant is also used as a softener and as a secondary active delivery agent in many personal care products. Owing to its very high molecular weight, it exhibits a strong adherence to fabric. Stains containing colorless guar gum can appear removed in the wash, but often the guar gum is left behind. Particulates arising from wear of the garment or coming from a second wash can bind to the gum and show up as stains. Mannaway®, which originates from Bacillus, was shown to effectively cleave the β-1,4-linkages between mannose units in guar, thereby dramatically reducing the “reappearing stain” phenomenon.
Figure 4 shows an example of the effectiveness of Mannaway® at removing guar gum from fabric. The images were taken by confocal fluorescence microscopy by flowing a solution of liquid Tide® containing Mannaway® over a piece of cotton textile stained with fluorescently labeled guar gum. The bright spots correspond to the guar gum. After the wash, the guar gum is almost completely removed.

In the laundry context, Mannaway® has been shown to provide a broad stain-removal profile directly on stains containing guar gum, as well as whiteness benefits on other items, as a result of its ability to effectively degrade guar gum and prevent its redeposition. As alluded to above, the broad benefit profile is likely due to the pervasiveness of guar gum use in the food and personal care industries. Figure 5 shows an example of how Mannaway® can significantly improve the removal of a range of stains when using a liquid laundry detergent.

3.5 Future Perspectives

As detergent manufacturers continually work to grow sustainable market share, they often look to develop new products or product forms that provide clear, claimable benefits to consumers and that cannot be easily copied by competitors, i.e., a liquid detergent with bleach or improved fabric

![Fig. 4](image1.png)

**Fig. 4** Confocal fluorescence microscopy images showing the removal of fluorescently-labeled guar from cotton by Liquid Tide® containing Mannaway®. Figure kindly provided by Proctor & Gamble.

![Fig. 5](image2.png)

**Fig. 5** Stain-removal performance of Mannaway® on a range of different guar-containing stains. Figure kindly provided by Proctor & Gamble.