5

Practical aspects of handling enzymes

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5.1 Introduction

Processes for natural fibre-based fabrics have been developed using enzyme technology for the degradation of starch after weaving, the scouring of cotton fabric before dyeing, the removal of excess hydrogen peroxide before dyeing, modification of cotton fabric (finishing or biopolishing), production of Lyocell fabric, ageing of denim, modification of wool, degumming of silk and for the treatment of water effluent from textile production mills (refer to Chapters 3 and 6 for more extended descriptions). Today, research focus is even directed to the modification of synthetic fibres such as polyester and nylon.

The application of enzyme technology is very specifically targeted to a component (substrate) present in the fibre. In this way, the main characteristics of a fibre are maintained, instead of what is experienced often with chemical processes where the fibres are modified rather unspecifically. For instance, in the enzymatic scouring process with pectinase the enzymatic action is specifically targeted towards the pectin polymer, leaving the cellulose polymers unmodified.

Besides its effectiveness, enzyme technology is also preferred for its environmentally friendly character since no hazardous chemicals are used, unlike the situation in chemical processes, and the enzyme itself is also fully biodegradable. Owing to the ongoing integration of enzyme technology in an already large, and still increasing, number of partial processes, the textile production industry is shifting from a notoriously polluting industry to a more environmentally friendly one. Additionally, the textile area has been recognised as innovative.

Many people in the textile area and working with enzyme technology are textile engineers, having no, or only limited, biochemical background. Experience of handling a biocatalyst correctly is often not available in laboratories and at production sites. The incorporation of this more practical chapter is meant to compensate for this lack in knowledge concerning enzyme handling.

The main focus of this chapter concerns the enzyme's structure stability, the different possible inactivation processes and the methods available to stabilise the enzyme. Additionally, attention is given to handling enzymes in practical circumstances and to safety issues when working with this technology.

5.2 Enzyme activity

Enzymes are proteins specialised to catalyse (biological) reactions. They are among the most remarkable bioactive molecules known because of their extraordinary specificity (concerning both substrate and reaction) and catalytic power, which are superior to those of many synthetic catalysts. In general, a chemical reaction takes place when a reactant can pass the transition state wherein the reactant possesses enough energy to form or to break chemical bonds. The difference in energy between the initial and transition state of the reactant is lowered when the reaction is catalysed by an enzyme, owing to a transient combination with the reactant. In this way, an enzyme accelerates a chemical reaction by lowering the energy of activation (see also Chapter 3).

The chemical reaction takes place in the active site of the enzyme, a three-dimensional hole in the protein where the reactant can interact optimally with one of the amino acid side chains. The three-dimensional structure of the active site should be maintained for optimal catalytic power and substrate specificity. Or, to put it another way, conservation of the enzyme specificity and catalytic power, its stability, is realised by maintaining the three-dimensional structure of the enzyme itself and its active site in particular. The overall three-dimensional protein structure is dependent on the amino acids present in the protein and the sequence of those residues. Different substructures are distinguished: primary (sequence of amino acid residues in the polypeptide chain), secondary (regular recurring arrangement in space of the polypeptide chain along one dimension), tertiary (polypeptide folding in three dimensions) and quaternary (arrangement of two or more polypeptide chains in relation to the others to form one protein).

5.2.1 Maintaining the three-dimensional structure of a protein

Different interactions between the side chains of different amino acids are the basis of the conformation and stability of the enzyme. The following interactions can be distinguished:

- Hydrophobic interaction. Some amino acids have aliphatic (alanine, leucine, isoleucine, valine and proline) or aromatic (phenylalanine and

tryptophan) side chains, which can all be classified as hydrophobic. Those amino acids are often on the inside of the protein, away from the surface which is in contact with water. Those side chains are interacting under release of bound water with a subsequent increase in entropy (Edelstein, 1973). So, hydrophobic interaction is an entropy-driven process.

- Van der Waals interaction. Van der Waals interactions are caused by attractive forces between transient dipoles of uncharged atoms and are very weak. The forces operate at relatively long ranges.
- Hydrogen bonds. Hydrogen bonds (amide–carboxyl bonds in the basic polypeptide chain) contribute significantly to the stabilisation of the secondary structures of a protein, i.e. α-helici and β-sheets. Additionally, other hydrogen bonds are also important for the stability of a protein. Amino acids that can act as donors of hydrogen atoms for the formation of hydrogen bonds are the hydroxyl group-containing serine, threonine and tyrosine, and the amino group-containing asparagine and glutamine. Asparagine and glutamine can also act as hydrogen acceptors. Many of these residues in various combinations occur in the diversity of hydrogen bonds throughout the protein structure.
- Electrostatic interactions. Electrostatic ion-pair interactions often play an important role in relation to the stability of a protein. Amino acids involved are the carboxyl side chain-containing aspartic and glutamic acids and the amino side chain-containing lysine, arginine and also histidine.
- Ion coordination interaction. As a variant of electrostatic interaction, some proteins contain a cation (examples are Fe^{2+}, Mg^{2+} and especially Ca^{2+}) which functions as a structure coordinator by interaction particularly with aspartic and glutamic acids. The presence of such a coordinating ion is essential in most cases. Upon removal by extraction with a chelator, most enzymes are inactivated immediately.
- Covalent coupling of two cysteine amino acids. Two cysteine amino acids from different places in the primary structure of the polypeptide chain can be coupled under the formation of a disulphide bond or bridge. Because this coupling is covalent, it has a major impact in relation to the maintenance of the biological active conformation of an enzyme.

The three-dimensional structure of an enzyme is not a stiff conformation, but more a dynamic equilibrium wherein each interaction, as mentioned before, plays its own specific role. An impact on the three-dimensional structure can be expected when the environmental situation of an enzyme changes. As an example, when the pH of an enzyme solution is shifted, one can expect an influence on the strength of the electrostatic interactions (the degree of ionisation will be shifted), which may have an impact on struc-

ture and therefore on the activity of the enzyme. In practice, the impact will be small, if not negligible, when such a pH shift is limited, but can be dramatically increased in a situation of extreme shift.

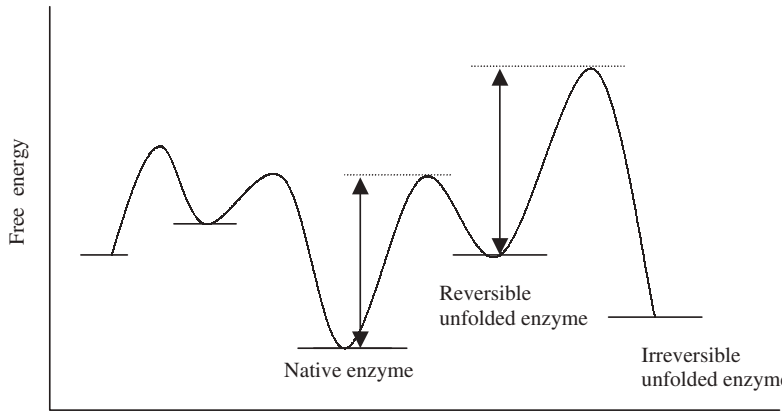
In a situation of increased temperature, the enzyme molecules are in a higher energetic state, resulting in increased vibrations between atomic bonds. Different structure-determining interactions (the van der Waals and hydrogen interactions) will be influenced. Raising the temperature beyond a certain threshold value, which is different for each type of enzyme, will lead to loss of interactions and, therefore, protein unfolding and loss of catalytic activity.

5.2.2 Enzyme inactivation

Since the catalytic power of an enzyme depends on a correct three-dimensional conformation of protein, the active site part in particular, the biocatalyst is rather sensitive to influences from its environment which have an impact on this conformation. Changes in conformation of the enzyme often, but not always, result in reduction of its catalytic power towards zero.

Because of the complexity of an enzyme protein, many protein conformations or foldings are possible, all exhibiting their own level of free energy. Only one conformation possesses the lowest level of free energy, the native enzyme conformation. In general, inactivation of an enzyme by protein unfolding can be described as a two-step process in which the irreversible conformation at the end is reached via a transient reversible conformational change. This reversible intermediate conformation is more labile and therefore easily transforms to a more unfolded, irreversible conformation, a situation without any catalytic power. The increased lability of the transient intermediate conformation can be best explained by the increased level of free energy, see Fig. 5.1. Owing to this increased free energy level, the required free energy of activation to transfer to an inactivated enzyme conformation is lower. The reversed transfer from the inactive conformation back to the native one is blocked by the height of the energy barrier, explaining the irreversible character of this conformational change. The energy barrier for a direct transfer from the native enzyme conformation to the irreversible unfolded enzyme conformation behaves similarly.

Enzyme inactivation by protein denaturation (unfolding), aggregation (interaction between different enzyme molecules) and precipitation (protein becomes water-insoluble upon conformational change) always proceeds via an intermediate, reversible unfolded conformation. Enzyme inactivation processes upon chemical modification (covalently) may proceed via such an intermediate conformation, but direct modification of the native protein is also possible. In the latter case, protein modification will be limited towards the amino acid residues exposed to its surface.



5.1 Protein unfolding in relation to the energetic state of the conformation. Arrows indicate level of activation energy required for each transition from native to irreversible unfolded enzyme.

There are many circumstances in which enzymes are irreversibly inactivated. Although this statement suggests that industrial application of enzyme technology is not realistic, the opposite is valid. A prerequisite to obtaining good enzyme technology performance is that certain circumstances have to be avoided. The following is an enumeration of what may be experienced in practice when setting different parameters and what has to be avoided.

5.2.2.1 Temperature

As in most chemical reactions, the rate of enzyme-catalysed reactions generally increases with temperature within the temperature range in which the enzyme is stable and retains full activity. The rate of most enzymatic reactions roughly doubles for each 10°C rise in temperature. The exact rate improvement varies from enzyme to enzyme and is dependent on the energy of activation of the catalysed reaction (the height of the energy barrier to the transition state). The activity versus temperature curves, as published by the enzyme manufacturers in the delivered data sheets, always show a temperature optimum. This is due to the fact that the enzyme is denaturated (unfolded) above a certain threshold temperature (each enzyme exhibits its own threshold value). The apparent temperature optimum is thus the result of two processes: the increase in reaction rate with temperature and the increasing rate of thermal denaturation of the

enzyme above a critical temperature value. Mostly, the increase in denaturation rate is very high beyond the critical temperature value. The published temperature optimum is also dependent on the conditions of the test. Measurement time is an important parameter: the shorter the incubation time, the lower the possible impact of enzyme denaturation. The use of different types of substrate (reaction reactant) may also have an impact on the observed temperature optimum as a consequence of substrate binding (stabilisation factor). Therefore, determination of the optimal temperature under identical conditions to the application itself is recommended. If long incubation times are required, it is wise to fix the temperature approximately 5°C lower than the published optimum.

5.2.2.2 pH

Enzymes have a characteristic pH at which their activity is maximal. This maximum can be in the acidic, neutral or alkaline pH range. Often a bell-shaped activity–pH curve is observed. The exact shape however can vary considerably in form from enzyme to enzyme. The pH–activity relationship of any given enzyme depends on the acid–base behaviour of both enzyme and substrate. One can imagine that upon shift of the pH, the electrostatic interactions may change within the enzyme with subsequent possible impact on its three-dimensional conformation and thus its activity. Likewise this is true when the substrate binding to the enzyme is driven/influenced by electrostatic interactions.

The shape of the pH–activity profile usually also varies with substrate concentration, since the affinity constant K_M of most enzymes changes with pH. Such a pH–activity curve is therefore most meaningful for industrial application if the enzyme has been kept saturated with the substrate at all the pH values tested.

Most enzymes have a certain pH tolerance range, often wider than one pH unit, in which their activity is 80–100% of its maximum. Within this range, the pH may shift without major consequence for the enzymatic activity. Extreme pH values far from the enzyme's maximum in the pH–activity profile should be avoided at all times. These values have a major impact on the electrostatic interactions within the enzyme, leading to irreversible protein unfolding and thus inactivation.

5.2.2.3 Surfactant

When dealing with water-insoluble substrates, as is often the case in the textile production industry, surfactants are used to improve the enzyme–substrate interaction. There are four major classes of surfactants:

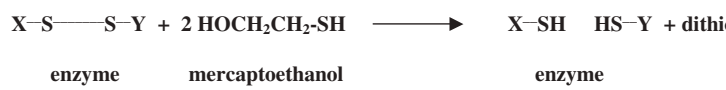
anionic, cationic, zwitterionic and non-ionic, all containing a more or less apolar tail or piece in the middle. All the charged surfactants can interact with the charged side chains of amino acids on the protein surface. Upon electrostatic interaction between surfactant and enzyme, a process may be initiated wherein the apolar part of the surfactant penetrates to the interior of the protein in order to accomplish hydrophobic interaction. The consequence of such interactions may be a reversible conformational change in the first instance, followed by an irreversible one. In this way, the presence of a surfactant, especially the anionic and cationic ones, results in inactivation of the enzyme. The compatibility of any surfactant has therefore to be checked prior to use. In general, the compatibility with enzymes of non-ionic surfactants is highest, whereas that for anionic is lowest. It has to be said that if one representative of a category is not compatible with an enzyme, that does not mean that another representative of that category is also incompatible. The type of apolar tail and especially the length of the apolar tail are also of importance if the above-mentioned inactivation process occurs. If an enzymatic process is developed wherein incorporation of a surfactant is required for optimal performance, a compatibility study is recommended. If no compatible surfactant can be identified, a compatible enzyme of different origin may be used.

5.2.2.4 Chelator

As mentioned in Section 5.2.1, some enzymes contain a cation to coordinate the three-dimensional structure of the protein or to act as an essential cofactor for the catalytic reaction. When such an enzyme is used, the application of strong chelators has to be avoided in order to prevent extraction of this essential ion. When a chelator is desired for a certain process, one can look for a milder variant, which still fulfills the job. As an alternative, one can look for an enzyme without such a coordinating ion or an enzyme, which binds the cation more strongly and therefore cannot be extracted by the desired chelator any more. Information concerning the presence of such a cation can be obtained from the manufacturer.

5.2.2.5 Reducing agent

When operating a process with an enzyme containing disulphide bond(s), the presence of a reducing agent should be avoided. Normally, no reducing agents are used in the processes applied in the textile production industry. Nevertheless, when a reducing agent is present, the SH groups of the cysteine residues are regenerated, breaking the disulphide bond (Fig. 5.2). Upon breakage of this disulphide bond, the three-dimensional structure is no longer fixed by this covalent bonding. Unfolding of the protein may



5.2 Breakage of the disulphide bond in an enzyme via reduction by a reducing agent, i.e. mercaptoethanol.

occur, leading to inactivation of the enzyme. In such a situation an alternative enzyme without disulphide bonding has to be used.

5.2.2.6 Oxidising agent

Several amino acid residues are sensitive towards oxidation in the presence of a bleaching agent. First of all there are the sulphur-containing cysteine and methionine residues. Second, the aromatic residues tryptophan, tyrosine and histidine are also sensitive to oxidation, although to a much lower level. In a protein, those residues are oxidised when they are accessible to the bleaching agent: on the surface or in the active site. Oxidation of one of these residues in the active site may prevent adequate substrate binding and reduces the enzymatic activity towards zero, although the active site machinery and the three-dimensional structure of the enzyme is still intact. Oxidation of a surface amino acid residue may lead to conformational changes, with enzyme inactivation as the end result. The sensitive residues located further into the interior of the protein are protected from oxidation unless a reversible conformational change makes them accessible to the bleaching compound.

When an enzyme appears to be sensitive towards oxidation in the application, genetic engineering can be used as the preferred tool to replace such residue for a non-oxidation sensitive residue (Misset, 1993). In practice, the enzyme suppliers will do this. The user of enzyme technology can search for an alternative commercially available enzyme that is bleach resistant in relation to its activity.

5.2.2.7 Protease contamination

Enzymes are proteins and can therefore function as a substrate for protease enzymes. Upon (partial) degradation of the enzyme, its three-dimensional structure can collapse and inactivation will occur. In the case of protease enzyme application, autodigestion can occur wherein one protease molecule degrades another one. The addition of calcium may suppress this phenomenon to a certain extent. Contamination in the process by a protease may occur via air dust, when protease incubation has previously been executed, or by contamination with microorganisms producing extracellular proteases. In both situations, water-based cleaning of the equipment at a

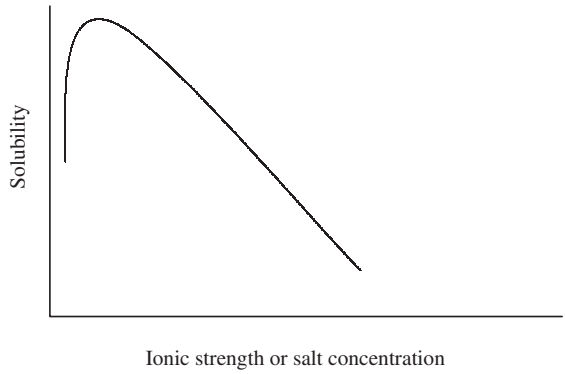
temperature beyond the enzyme stability threshold value is the preferred method. This can be combined with a cleaning solution exhibiting an extreme low or high pH value, conditions wherein most enzymes have drastically reduced stability. Inactivation of the proteolytic activity by chemical modification of the protease is an alternative (see Section 5.2.2.11).

5.2.2.8 Precipitation

Protein solubility in water depends on the pH of the buffer system. As mentioned before, a substantial part of amino acids with hydrophilic side chain residues are situated at the surface of the protein, for example the basic and acidic ones. The overall electric charge of the protein at a certain pH depends on the composition of those amino acids at its surface. The pH at which a protein is least soluble, and will precipitate most easily, is its iso-electric pH, defined as that pH at which the molecule has no net electric charge. At this pH, and in the range closely surrounding it (roughly a 0.5 pH unit width, although this is different for each protein), the solubility of the protein is minimal. Outside of this range, the solubility increases drastically.

In a situation where an enzyme is not fully soluble at the required application dosage, precipitation of the enzyme will occur, with consequent loss of enzymatic activity in solution. To prevent such a situation, a pH shift of several tenths of a pH unit is recommended. Depending on the pH–activity curve of the particular enzyme, the pH shift has to be realised at a lower or higher value.

An alternative practical solution of the problem of enzyme precipitation is the addition of salt, since the presence of some salt will increase the solubility of proteins. This phenomenon is called salting-in (Lehninger, 1975) and is also valid around the iso-electric point of an enzyme. A salt concentration in the millimolar range (20–50 mM is sufficient in most cases) will increase the solubility of a protein by a factor beyond 10 for most proteins. Different types of salts can be used. In general, the salts of divalent ions, such as $MgCl_2$ and $(NH_4)_2SO_4$, are far more effective than salts of monovalent ions such as NaCl, NH_4Cl and KCl. It is recommended that the impact of salt addition on enzyme performance be evaluated with respect to enzyme precipitation. Besides this salting-in principle, there is also a salting-out effect: when the ionic strength (salt concentration) is increased further, the solubility of the protein begins to decrease (see Fig. 5.3). At sufficiently high salt concentration, an enzyme protein may be almost completely precipitated from the solution. The mechanism or physicochemical basis of this phenomenon is rather complex, but one factor is that high salt concentrations may remove water of hydration from the protein, resulting in reduced solubility. In this way precipitated enzymes retain their native conformation and can be dissolved again, usually with no loss of activity. The salting-out principle may be used to recover the enzyme after applica-



5.3 Impact of salt concentration (ionic strength) on protein solubility.

tion for re-use. Ammonium sulphate is the preferred salt because of its high solubility in water and therefore the possibility of precipitating the enzyme at almost 100%. The required salt concentration is within the molar range.

5.2.2.9 Aggregation

Normally, in a protein solution all molecules have an identical net charge of the same sign. The molecules therefore repel each other preventing coalescence of single molecules into aggregates. In a situation where a protein shows hardly any net charge, i.e. near or at the iso-electric point of the protein, repellence may not be high enough and aggregates can be formed. Depending on the type of aggregates formed, the enzyme may become partly reversibly inactivated with a significant drop in performance in the application. As in the situation of enzyme precipitation, a pH shift can be very effective in preventing this situation.

Enzyme aggregation may also occur when dealing with a protein with organised positive and negative charged side chain residues on its surface, resulting in distinguishable areas with opposite overall net charge. As a result of electrostatic interaction, dimers, trimers or tetramers may be formed with a decrease of enzyme activity as a possible consequence. In such a situation, the addition of small amounts of salt can prevent the formation of these paired enzyme molecules.

5.2.2.10 Shear force

The correct three-dimensional structure of an enzyme for enzymatic activity should be defined by the total number of interactions present in such protein (see Section 5.2.1). The overall level of interactions within such enzyme determines its sensitivity towards external added energy such as temperature. Energy addition in the form of applied shear force (stirring

for instance) may also have an impact on maintaining the enzymatic activity. Shear force may initiate reversible protein unfolding first, followed by subsequent irreversible conformational change, leading to denaturation and inactivation of the enzyme. Enzymes exhibiting high turnover numbers often have a relatively low resistance against shear force, as do enzymes originating from cold-loving microorganisms. Equipment used in the textile production industry, which operates in the batch mode, has a relatively high level of intrinsic shear force compared to continuous operating equipment. In a situation where the enzyme is inactivated as a consequence of applied shear force, one can try to lower the level of shear force or to exchange the enzyme for another one of the same type but originating from a different microorganism.

5.2.2.11 Chemical modification

Chemical modification of enzymes leads to covalent changes in or on the surface of the protein, often with a shift in activity or enzymatic part-inactivation (formation of a cripple enzyme) as a consequence. Examples of covalent enzyme modification have already been mentioned: oxidation, reduction and proteolysis. Other examples are deamidation, Maillard reactions and modification of an active site residue resulting in the blockade of the biocatalyst. As an example of an active site modification, the phosphorylation of an active site serine should be mentioned (Lehninger, 1975): the hydroxyl group of an active site serine, as present in, among others, trypsin, chymotrypsin and several esterases, becomes phosphorylated upon reaction with di-isopropylphosphofluoridate. This chemical modification reduces the enzymatic activity towards zero, although the three-dimensional structure is still intact. However, the substrate is not subsequently able to bind in a correct way in the active site in order to undergo the catalytic reaction. Situations in which enzyme modification can occur, no matter what the kind of modification, should be avoided at all times. There is no simple handling possible to overcome this modification except to avoid the situation wherein such a modification agent is present.

In one particular circumstance beneficial use can be made of the active site modification. In a situation of protease contamination, the proteolytic activity may be reduced specifically by modification of its active site. A prerequisite is that the applicable enzyme should have a catalytic site different from that of the protease.

5.2.2.12 General remarks

During application, inactivation of the biocatalyst is undesirable. Subsequently, however, the enzyme frequently has to be inactivated to prevent

undesirable prolonged enzymatic activity, such as in the case of ongoing cellulose material destruction by cellulase enzymes. The easiest method of effective enzyme inactivation, valid for most commercially available enzymes, is a combined treatment of extreme pH value at high temperature (80 to 90°C) for a limited amount of time (5 to 10 minutes).

A second point is that enzyme inactivation is not identical to reversible enzyme inhibition. Although in both cases no enzymatic activity is observed, the underlying basis differs fundamentally. In the situation of reversible enzyme inhibition, the inhibitor competes with the substrate to bind in the active site of the enzyme. Upon inhibitor binding, the inhibitor remains unchanged, while the enzyme–inhibitor complex prevents substrate modification (enzyme inactivation). However, the enzyme remains active, in principle; upon removal of the inhibitor, for example by dilution, the enzyme returns to full activity. Inhibition deals with competition between a substrate and a non-enzymatic sensitive substrate analogue, while inactivation deals with modification of the enzyme itself.

5.3 Stabilisation of enzymatic activity

Enzymes can be isolated for use from many natural sources. However, for industrial application, the required enzyme quantities are such that they have to be produced artificially. This is done by fermentation using microorganisms containing the gene(s) encoding the desired enzyme. The industrial production of an enzyme can be segmented into three major processing stages:

1. synthesis of the enzyme by fermentation
2. downstream processing of the fermentation broth
3. formulation of the crude enzyme product.

In the fermentation phase, the microorganisms are multiplied and induced for production and excretion of the enzyme product into the fermentation medium. In the subsequent downstreaming process the enzyme product is isolated from the broth and partially purified. Often common laboratory protein purification methods are used which have been scaled up to industrial level. Techniques used include precipitation, (ultra)filtration, various chromatography techniques and (spray) drying. The crude enzyme product derived from this downstreaming process can be either a liquid or a solid preparation. After isolation, the crude enzyme product is turned into the final enzyme product via formulation. Formulation is executed for three reasons (i) to obtain a dosage form which allows (relative) easy handling during application (ii) to show optimal performance in the application and (iii) to ensure maximal enzyme stability. The final enzyme product is stored at the production facility before distribution.

Table 5.1 Indication of typical enzyme inactivation at each life cycle stage and the duration of each stage

Life cycle stage	Duration	Typical inactivation (%)
Fermentation	Hours–days	5 to 15
Downstream processing	Hours–days	10 to 25
Formulation	Minutes–hours	3 to 10
Storage and distribution	Weeks–months	5 to 20
Application	Minutes–days	10 to 50

At the customer's site the product will be stored again, before use in the application.

In all stages of the enzyme's life cycle, the enzyme should be stable. In practice, 100% stability will not be obtained at certain stages, but inactivation of the enzyme product should be minimised as much as possible by use of all means available. In Table 5.1 typical inactivation percentages are given for each life cycle stage together with an indication of the duration of each stage. Actual inactivation percentages will be different for each enzyme, but the data in Table 5.1 give a good representative impression of how stable commercial industrial enzymes are. Inactivation already occurs in the fermentation stage as well as the accumulation or production of enzyme, as a consequence, among other things, of temperature (approximately 30–40°C) and proteolysis. The largest percentages of inactivation are seen in the application stage, especially when an enzyme will be used once in a fixed time frame and in the downstreaming stage where conditions harmful to the enzyme can be applied regularly. The relatively low percentage of enzyme inactivation during storage and distribution is striking, although the time spanned in this stage is the greatest of all.

Enzyme stability has a crucial impact on the economics of both enzyme production (supplier) and application (customer). It is therefore not surprising that major attention has been focussed on this aspect, and in particular on the formulation of the enzyme. For the customer, only two stabilities are of importance, the storage and operational stability. The storage stability and the safety issues (aerosol formation, see Section 5.5) around enzyme products are both of interest to the supplier as well as to the customer. Formulation of the crude enzyme product is applied to maximise both stability and safe handling.

5.3.1 Storage stability

An enzyme product can be delivered as a solid or liquid. In turn, a solid enzyme product can be purchased as a powder or in granulated form. The

Table 5.2 Parameters which have major impact on the storage stability of enzyme, in both solid and liquid form

Solid	Liquid
Humidity/water content	Water activity
Temperature	Temperature
	Presence of harmful additives

former physical form is less favoured, since the chance of dust formation (safety issue for employees, see Section 5.5) is substantially higher than when the enzyme is granulated.

The main parameters of importance for maximal shelf (storage) stability are summarised in Table 5.2 for both a liquid and a solid enzyme preparation. For the enzyme preparation in liquid form, the water activity is of major importance with respect to microbial hygiene. Since enzymes are natural proteins, they are biodegradable and readily used as nutrients for microbial growth. This microbial growth is dependent on water and if the conditions are favourable, the enzymes will be degraded and enzymatic activity destroyed by the microbes. The process will have an intrinsic acceleration rate since the growth of microbes will increase upon increasing the amount of digestible nutrients (partly degraded enzymes).

Water activity is of less importance for a solid enzyme product. However, a solid enzyme also contains some free water. Also the enzyme molecules contain a minimal amount of bound water (hydration), essential to maintain the active three-dimensional structure. If the solid enzyme product is stored in an environment with high humidity, the product will take up water to increase the level of hydration of the enzyme and the filler that exists in a formulated solid enzyme product. In a situation where a sufficiently large amount of water is bound to the enzyme, the enzyme may undergo structural rearrangement depending on the storage conditions, with inactivation of the enzyme as a consequence.

Temperature is a crucial storage parameter for both the solid and liquid enzyme product forms. By raising the temperature, the intrinsic energy level of enzyme molecules is also increased, with an increased level of vibration of atomic bonds within the molecules as a consequence. In the presence of a minimal amount of water (often a prerequisite) the rate of structural rearrangement is increased and thus the rate of inactivation.

Finally, for the liquid product form, the presence of potentially harmful additives is of major importance. If enough water is present, chemical reactions and interactions with, for example, a surfactant may occur that may have impact on the enzyme activity.

There are many principles or methods that may be used to achieve maximum enzyme stability either by strengthening the intrinsic stability or by minimising enzyme inactivation. The following is an enumeration of the most frequently used methods to achieve optimal storage stability.

5.3.1.1 Temperature

To start with the simplest way of decreasing the rate of inactivation, the storage temperature should be as low as possible in order to slow down all possible processes which lead to enzyme inactivation. For liquid-formulated enzyme products in particular, where enough water is present, this is an important tool to control the rate of inactivation. For this reason the enzyme supplier often recommends a maximum storage temperature. For solid enzyme products this is mostly 25°C (77°F) or below. For liquid product forms the recommended storage temperature depends on the enzyme in question. For some relatively unstable enzymes, the recommended storage temperature is below 4°C, while for others the same temperature as that valid for solid products is recommended. Therefore, the supplier's product datasheet should be read carefully directly upon delivery of the product. A general recommendation is that the storage temperature of enzymes in a liquid form should never be below 0°C, the freezing point of water. Upon crystallisation of water, the three-dimensional structure of the enzyme may be disrupted with consequences for its activity.

Exceeding the recommended storage temperature does not mean that the enzyme is fully inactivated directly. A short storage time at a temperature beyond the recommended one will raise the potential inactivation rate for the time being, but on an absolute level, the enzyme inactivation will still be limited and the enzyme product will still be valid for application. Nevertheless, storage for prolonged times beyond the recommended temperature should be avoided in order to prevent substantial loss in activity of the enzyme product.

5.3.1.2 Storage in closed containers

Another relatively simple way of preventing enzyme inactivation upon storage is to keep the enzyme in closed (plastic) containers. In this way, no water uptake can take place to initiate enzyme inactivation. Additionally, especially for the liquid enzyme products, no contamination with microorganisms that are producing and excreting proteases can occur with subsequent proteolysis of the enzyme.

5.3.1.3 pH

As is well known, every enzyme has an optimal pH for activity, as well as for stability. To store enzymes in the liquid form, the pH should be adjusted to the optimal stability region which guarantees a long shelf life. The pH can be set by the simple addition of a suitable acid, for example acetic acid or a weak buffer system. The buffer system should be weak because a pH shift should be realisable when, in the application, another pH level is required. If a choice is possible in relation to shelf life maximisation, a pH shift towards the acidic region is preferred since potential microbial growth is minimised. For maximal prevention of microbial growth, the pH should be as far in the acidic region as the chemical stability of the enzyme allows, because most bacteria will require more neutral conditions for growth. On the other hand, enzyme precipitation may begin when the pH is lowered too far. This situation has to be avoided even if the enzyme could tolerate it; in such a situation, a homogeneous enzyme dosage in the application is hard to realise, with potential consequences for the overall performance.

5.3.1.4 Reduction in water activity

As already mentioned the water activity of liquid enzyme products is of importance in relation to microbial growth in case of contamination and should be kept at a level which does not allow vigorous growth. Reduction in water activity can be realised by maintaining a high dry substance level and high osmotic pressure of the enzyme-containing liquid.

Normally, in an enzyme-containing liquid there is only 1–10% enzyme proteins. To prevent growth of contaminating microorganisms, a dry substance level of approximately 50% is required (Auterinen, 2002). Realisation of this percentage should be done, by addition of chemical additives, in such a way that the osmotic pressure of the liquid is increased as much as possible. The addition of mineral salts is the easiest way to increase the osmotic pressure. Any salt may be used for this purpose, but common salts such as NaCl are used most frequently. The amount of salt applicable has to be found experimentally for each particular enzyme since the enzyme may precipitate, either immediately or after some period of storage. There is a pH dependency of this precipitation behaviour of the enzyme. In practice, the salt concentration in a liquid enzyme preparation is between 5–10% of the weight of the final product.

A different general method of increasing the dry substance of the liquid enzyme product is to add sugars and/or sugar alcohols. The smaller the molecular sizes of the sugar, the higher the osmotic pressure it causes. Thus monosaccharides such as glucose and sorbitol are often used. In addition, sorbitol has been found to have good enzyme-stabilising characteristics. It

probably chemically stabilises the enzyme molecules owing to the presence of multiple hydroxyl groups that can support the maintenance of the three-dimensional enzyme protein structure by forming ionic bonds with it. Chemicals with sugar alcohol-type structures and low molecular weight are also used, for example glycerol, ethylene glycol and propylene glycol. The amounts of sugars and sugar alcohols together that can be found in liquid enzyme formulations vary between 10 to 40% of the weight of the final product. In theory, organic solvents such as ethanol, acetone, methanol and so on may also be used to reduce water activity. However, in practice they are hardly used.

5.3.1.5 Use of enzyme interactive additives

Stabilisation of the enzymatic activity during storage by means of conformation fixation may be realised by addition of agents which bind to the enzyme molecule. One example is the addition of a substrate or substrate analogue which binds at the active site of the enzyme thus maintaining the correct and active three-dimensional structure during storage. Owing to an environment of low water activity (in the case of hydrolytic enzymes where water is one of the reactants) and the low storage temperature, catalytic reaction in the situation with a real substrate proceeds very slowly. The protective effect of a substrate (analogue) will therefore be maintained for prolonged times. Upon application, the enzyme solution is diluted, reducing the possible impact of this protective agent on the enzyme's performance. The addition of different kinds of polymers, like polyvinylalcohol, polyvinylpyrrolidone and polyethylene glycol also causes enzyme stabilisation by binding to the enzyme surface. These types of interactive additives are used for the stabilisation of both liquid and formulated solid enzyme products.

5.3.1.6 Application of absorbers and/or scavengers

Addition of agents that bind water can be beneficial in maintaining a low level of water content. In a granulated solid enzyme product, such an agent can be of particular importance when the enzyme layer is coated with this agent. Granule-penetrating water molecules from the environment will be bound by such an agent, preventing interaction with the enzyme and possible conformational rearrangement. Enzyme stabilisation by this method will be temporary. When the water-binding capacity is exceeded, the protective character of such an agent is reduced to zero. Nevertheless, depending on the concentration used, such an agent can protect the enzyme activity during a certain time window.

Using the same methodology can prevent enzyme inactivation by other harmful agents, for example bleaching agents. In the case of bleaching agents, easy oxidising molecules (scavengers) can be used to surround the enzyme layer. In this way neutralisation of the bleaching agents occurs before the enzyme is reached.

5.3.1.7 Incorporation of salts

Calcium salts or ammonium sulphate may also be used to protect enzymatic activity during storage. In liquid formulations, calcium salts are used in concentrations of less than one molar. The exact stabilisation mechanism is not well understood, but probably coordination by calcium ions (electrostatic interactions) in one way or another will strengthen the enzyme protein structure. Ammonium sulphate is often used for salting-out proteins (see Section 5.2.2.8). In the precipitated form, enzymes are quite inert towards any agent or physical condition. Upon dilution after storage, the enzyme returns in solution and will show its original activity. In principle, ammonium sulphate precipitated enzyme can be stored in both solid and 'liquid' form. There is one potential disadvantage in applying this method: upon resolvation by dilution, a relatively large quantity of salt is present, which may have an impact on the enzyme performance. In this case, removal of the salt is required prior to application, and this can be achieved by use of size-exclusion chromatography or dialysation, for example.

5.3.1.8 Addition of microbicides

To prevent loss of enzymatic activity by contamination with, and growth of, microorganisms which produce proteases, the incorporation of microbicides is another option as well as reduction of water activity. The most frequently used microbicides for enzymes are salts of benzoic acid and sorbic acid. Both microbicides are most protective in a mild acidic medium: the benzoate at around pH 4–5, the sorbate at around pH 5–6. The amount of benzoate or sorbate required varies between 0.1 and 0.3% of the weight of the final product. Alternatively, the methyl-, ethyl- and propyl esters of benzoic acid, called parabens, may also be used. These agents are optimally protective in the more neutral pH regions 6–7. The main disadvantage of this class of agent is poor solubility in water. Propyl paraben can be added only at 100 ppm (parts per million), methyl paraben at around 1500 ppm. All mentioned microbicides are compatible with enzymes and normal additives in food and thus their use is very safe.

There are more non-food applicable microbicides and some of them may be very effective for protecting enzyme preparations from contamination during storage. Their compatibility with the enzymes used in textile

industrial processes has to be checked prior to incorporation in enzyme preparations.

5.3.1.9 Enzyme immobilisation

Fixation of the enzyme onto a carrier will have a stabilising effect in relation to its three-dimensional structure. In particular, multi-point attachment, fixation via several covalent bonds, often leads to a drastic increase in conformation structure stability and therefore activity stability. However, the immobilisation treatment itself normally already leads to an activity decrease of roughly 10 to 20%. If the immobilisation is on the surface as well as in the interior of the carrier, (substrate and product) mass transfer limitation may occur, depending on the carrier particle size. In a situation of mass transfer limitation, part of the enzymatic activity capacity will not be used in the application (lack of substrate availability) leading to an observed apparent inactivation of the enzyme. When there is prolonged inactivation of the enzyme at the outer areas of the carrier during application, the intact but initially unused enzyme at the more internal areas of the carrier becomes active when the substrate reaches it after passing inactivated enzyme at the outer sites. Finally, immobilisation will raise the cost price of the enzyme system. Therefore, in practice, this technique for enzyme stabilisation during storage is not used.

Nevertheless, the technique is powerful in those circumstances where the enzyme can be reused in the application many times in order to reduce enzyme costs. Examples, where enzyme immobilisation is applied successfully are in the conversion of glucose into fructose for the production of high-fructose corn syrup by use of immobilised glucose isomerase, and in the production of 6-aminopenicillinic acid by use of immobilised penicillin acylase. Potential is identified for use of immobilised enzyme systems in the textile production sector in wastewater treatment and in the removal of excess hydrogen peroxide by use of immobilised catalase.

5.3.1.10 Genetic engineering

A different way of stabilising enzyme activity is to modify the enzyme protein itself by means of genetic engineering. Despite the fact that the structure–stability relationship is not always known, when the amino acid that causes instability is identified, site-directed genetic engineering could be applied. If the amino acid is not identified, random mutagenesis is a possibility. In this case many different mutants are generated randomly and screened for improved stability. Compared with site-directed mutagenesis, random mutagenesis is quite laborious and is therefore less preferred. In a situation where it is known which amino acid residue is modified upon inac-

tivation, this amino acid may be replaced by, in principle, any other one. In practice, substitution by different amino acids often has to be studied, since not only is the modification of that particular amino acid blocked by its replacement, but also a shift in both the enzymatic activity, and, more importantly, performance is initiated. In addition, incorporation of another amino acid may result in incorrect folding of the protein, resulting in a total loss of catalytic activity. Nevertheless, most trials of modifying an enzyme through replacement by a particular sensitive amino acid result in improved stability while maintaining the performance as it was with the original wild-type enzyme. The major benefit of this method is that once the genetic code for an enzyme has been changed, production will be like that of the wild-type enzyme, and its behaviour in the downstreaming process is often similar. So the economics of producing a mutant enzyme will be identical to that of the wild-type enzyme. Performance in an application and enzymatic activity using a particular substrate are not correlated. Therefore, performance of generated mutants has to be evaluated instead of an activity test. As an example, the successful improvement of bleach stability (against hydrogen peroxide) of a protease has been realised upon replacement of the bleach-sensitive methionine by either a serine or glutamine (Misset, 1993).

5.3.1.11 Chemical modification

A final possibility for improving stability of enzymes is chemical modification. This can be a one- or two-point (internal crosslinking) modification. As an example for a one-point modification the modification with glyoxal can be mentioned. A well-known crosslinking procedure is the reaction with glutaraldehyde where the aldehyde groups at both ends of the molecule react with the amino groups in the side chain of amino acids. Chemical modification requires reactive amino acids, which are the basic, acidic, alcoholic, aromatic and the sulphur-containing ones. This modification is not selective because the reagent will modify all surface-exposed amino acids of a given type. Although improved stabilities may be realised, there are major drawbacks to this method. First, the enzymatic activity and enzyme performance may be modified (often reduced) upon chemical modification, resulting in the need for increased enzyme dosage in the application. Second, the chemical modification step has to become an integral part of the enzyme production process and will increase the cost of the enzyme product. For these reasons and the technical limitations, chemical modification is often not attractive to apply in practice.

In the textile production industry, enzyme technology has already been in use for some years. A striking observation is that roughly 70–80% of

the enzymes used in this industry are in non-formulated powder form (Auterinen, 2002). Although enzyme formulation is not essential for stability *a priori*, it is essential for dust prevention (safety issue, see Section 5.5). Handling enzyme powder instead of formulated enzyme product is more dangerous for employees because the chance of dust formation and subsequent enzyme inhalation is larger. Since the use of enzyme technology is growing in the textile area, both in quantity and number of applications, more attention should be given to safety of handling and therefore to the use of formulated products. In another industrial area with even more experience of enzyme application, i.e. the detergent or cleaning area, this aspect has been given full attention. In this sector high-tech formulations have been developed in order to incorporate enzymes in a safe-handling and enzyme-stable way into detergents, which are harmful environments for enzymes with high pH, anionic surfactants and bleaching agents. Whereas Novozymes has developed the T-granulate formulation (T stands for tough), the other major enzyme supplier, Genencor International, applies its own developed Enzoguard® technology. The T-granulate, a more or less spherical particle, contains a core wherein the enzyme is incorporated. This core consists of cellulose fibres and is encapsulated by an inert coating. The material of the core and its coating give the granulate elastic properties, which makes it resistant to mechanical crushing and the release of enzyme dust.

The Enzoguard® technology is based on a different principle. By applying fluid-bed granulation technology, a multitude of layers is applied around a small spherical prefabricated core. The first layer on top of the core, containing a sugar, is the enzyme-containing layer. Owing to its concentration within a small spherical layer around the core, instead of within the core, the enzyme will dissolve in water very fast. The second layer around the enzyme layer has a scavenger function and contains chemicals to protect the enzyme from inactivation by chemicals (bleaching agents) and water (humidity) coming from outside. The outside layer is a functional coating developed and applied for optimal storage stability of the enzymatic activity.

Both formulations, although different, show substantially improved enzyme storage stability, very well reduced levels of potential dust formation upon handling and good dissolution profiles in water. It may function as a good example in the textile area of how to formulate enzyme powders for improving safe handling and storage stability.

5.3.2 Operational stability

As well as during storage, the enzyme may also be inactivated during application. Although the time window for application is relatively small compared with that of storage, the rate of inactivation is often the opposite. As in the situation with storage stability, there are some methods that max-

imise enzyme stabilisation during application. First of all, in application the goal should be the best obtainable enzymatic performance at a certain fixed cost level, and not enzyme stability *per se*. In a situation where the enzyme performance can be obtained rather easily, there is or may be some space left to trigger some application parameters towards improved enzyme stability. Even in a situation where the enzyme is applied once with subsequent inactivation, an improved stability can be beneficial through a reduced level of required enzyme dosage.

Certain methods that can be used for enzyme storage stability improvement are also valid for increasing its operational stability. These methods are:

- additive dosage
- immobilisation
- genetic engineering
- chemical modification.

The last three methods, used for improvement of storage stability, will also have a positive impact on operational stability. For the first method, it should be kept in mind that additives, dosed in the enzyme formulation for storage stability improvement, will lose their stabilising effect in the application as a consequence of dilution of both enzyme and stabiliser. Besides the above mentioned methods, there are some additional ones, which are enumerated below.

5.3.2.1 Temperature

In principle, decreasing the operational application temperature will lead to improved enzyme stability. Equally, the enzymatic activity will also be lowered. If the factor time is of no importance, one may screen for an optimal temperature at which the process costs are minimised (a balance between costs of chemicals and energy required). In practice, noticeable enzyme inactivation starts after passing an enzyme-particular threshold temperature. The optimal temperature recommended by the enzyme supplier is mostly a value just below this threshold value. In many screening tests for optimal operation temperature one will end up near the recommended temperatures when the intrinsic energy content of the enzyme is the driving force for inactivation (denaturation of the protein). In those situations where the enzyme inactivation is induced, for example by interaction with a compound (among other things this can be a charged surfactant) present in the application, the screened optimal temperature may be substantially different (lower) from the recommended temperature. The opposite situation can also occur: when an enzyme is stabilised by interaction with a component (among other things this can be a polymer), the threshold value at which enzyme inactivation starts can be shifted

towards higher temperature values. In general, the temperature is not a parameter that is used for operational stability improvement.

5.3.2.2 pH

As mentioned in Section 5.3.1, the pH value for maximal activity is often not the value at which the enzyme shows its maximal stability. As stated in the beginning of this section, the focus in the application should be on enzyme performance, often, but not always, coupled with activity. In an application situation where the enzyme is used once and inactivated afterwards, the pH should be chosen normally for (near) maximal enzymatic activity instead of stability. However, when an enzyme-destabilising interaction with another required ingredient in the application is present, for example interaction with a charged (anionic or cationic) surfactant, it may be valuable to choose a pH for maximal enzyme stability. The rate of interaction-induced enzymatic inactivation can by very high with huge impact on the performance as consequence. Electrostatic interaction that leads to enzyme inactivation by unfolding is pH dependent. Thus, a shift in operation pH value away from that of maximal activity may increase the overall performance as a consequence of reduced enzymatic inactivation rate.

In a situation where the enzyme is reused, the enzyme stability during application becomes more important. In such situations, the pH has to be set more in relation to maximal half-life time (the time in which the enzymatic activity has been decreased by 50% while operating under application conditions). An eventual decrease in enzymatic activity can be compensated by a higher dosage or by increasing incubation time. The exact pH setting should be done in such a way that a minimum value of the overall processing costs is realised.

5.3.2.3 Substrate concentration

When an enzyme and its substrate are brought together, they initially form an enzyme—substrate complex prior to the enzymatic reaction to generate product. During the catalysed reaction, this complex is in equilibrium with the individual substrate and enzyme molecules. When increasing the substrate concentration, this equilibrium is shifted towards the enzyme—substrate complex form. In such a situation of increased substrate concentration, more enzyme will be present in the enzyme—substrate complex form. As already mentioned in Section 5.3.1.5, the binding of a substrate molecule in the active site of an enzyme may stabilise the enzyme by preventing protein unfolding. Working in the application with a raised substrate concentration will therefore stabilise the enzyme, especially at the start of enzymatic substrate conversion where high amounts of substrate molecules are still present in the system.

This method for improving the operational stability of enzymes is of less importance for the textile industry because, in textile production processes, the substrate will often be either the fabric itself or the substrate concentration will be fixed, as in the situation where the desizing procedure makes use of α-amylase. Manipulation of the substrate concentration in these situations is not possible. It might, however, be a method for the future treatment of wastewater by enzymes.

5.3.2.4 Additives

As mentioned at the start of this section, additives may have a positive effect on enzyme operational stability as well as the effect they can have on storage stability. Addition of ions in the application may have an enzyme-stabilising effect under application conditions (diluted enzyme condition) owing to electrostatic interaction, without any negative impact on its performance. As an example in the textile industry, the optimal use of many α-amylases in the desizing process can be achieved in water with a hardness of around 15°dH instead of using demineralised water. Similarly addition of other components such as diverse polymers, e.g. polyvinylalcohol or polyvinylpyrrolidone and surfactants (especially the non-ionic ones) may have a stabilising effect that is due to interaction with the enzyme, thus preventing protein unfolding.

5.4 Handling of enzymes

In principle, an enzyme should be handled as a chemical with all the same precautions necessary for handling chemicals. In addition, enzymes are bioactive as long as their three-dimensional structure is maintained. To secure this structure in solution, some additional precautions have to be taken, such as avoiding extreme circumstances concerning pH and temperature, and incompatibility with other processing agents.

For general handling of enzymes, the handling guide received from the enzyme supplier should be followed. If a particular enzyme requires a different handling procedure, this will be notified in the enzyme datasheet or in a separate document. In this section some handling of enzymes in different situations is discussed.

5.4.1 Handling enzymes in batch and continuous equipment

In general, application of enzyme technology in the textile production industry will proceed in batch or continuous operating equipment. The application and handling of enzymes in both types of equipment will differ somewhat as a consequence of the process flow within that equipment. For

both situations, the preparation of the ready-to-use enzyme solution will be the same, although the final enzyme concentration may differ. The method for preparing such solutions is described in Section 5.4.2.

5.4.1.1 Batch equipment

In batch equipment, for instance in a Jet construction, a limited amount of fabric is deposited in the equipment and treated in a process where a relatively high level of shear force is applied, inherent to the equipment used. The fabric has a high level of contact with the water medium, while the flow through the fabric (mass transfer) is on a relatively high level. The processing time in such equipment can be varied very easily as it can be set on hold for a while to check the performance level.

In this type of system, the enzyme solution will be introduced to the process when all other ingredients, together with the fabric, are already there. The required enzyme dosage, as determined in previous labscale experiments, can be applied at the start of the application and the application process is executed using parameters compatible with the enzymatic activity. The stirring device is activated directly upon enzyme dosage and heating the incubation medium to the required temperature application level is started. The temperature of the ready-to-use enzyme solution itself should preferably be between 5 and 20°C. During the process, the pH should be checked regularly in order to maintain the correct pH tolerance range. If correction is required, this can be realised using one of the methods described in the next section. If the development of performance lags behind previous experience, several possibilities are available to reach the desired performance if the origin of the lower performance is related to enzymatic activity. The simplest solutions are extending the incubation time and raising the temperature. The latter option should be chosen only in the last stage of the enzyme process, since the enzyme denaturation rate will be increased parallel to that of enzymatic activity. Additional enzyme dosing directly in the application is an alternative method.

After application, dosing an alkaline agent directly into the application medium, to raise the pH beyond 10 and raising the temperature to beyond 80°C with maximal heating capacity, can inactivate the enzyme. Since this will often take a couple of minutes, a duration time of 5 minutes at this elevated temperature will be enough to inactivate the enzyme.

Applying enzyme technology in the textile production industry is done most easily in equipment operating in the batch mode as a consequence of the easy way in which the different required process parameters can be established. There is only one parameter whose level has to be verified to maintain enzyme activity, the applicable shear force. Since the shear force

can be rather high is this equipment, it may be a driving force for enzyme inactivation by protein unfolding for those enzymes exhibiting a rather labile three-dimensional structure.

5.4.1.2 Continuous operating equipment

In a process operating in a continuous mode, the situation for enzyme application differs substantially from that in the batch mode. Here, the fabric substrate and enzyme medium are not normally in continuous contact during the whole process, but for a short time only. Wetted fabric with a transport velocity of 1 m/s or beyond is squeezed prior to passing through an enzyme bath. There, the fabric has to pick-up the enzyme or adsorb a film of enzyme-containing water. During incubation, the fabric may remain in a steamer or other temperature-conditioned environment. However, the incubation time is limited by the amount of fabric in such a compartment (limited capacity for fabric hold-up). If enzyme incubation occurs in the enzyme bath itself for reasons of, for instance, failure in pick-up of the enzyme by the fabric, the incubation time must be even shorter since the possibility of holding a substantial amount of fabric in the solution-containing compartment are even more limited. In the former situation, it is recommended that the volume of the enzyme bath be limited. Dosing of additional liquid enzyme solution from a cooled enzyme storage vessel can be carried out continuously while operating the process.

Monitoring the enzymatic activity may be a problem in a continuous operating enzymatic process. Monitoring is required in order to know the velocity of enzyme pick-up by the fabric and therefore to establish the enzyme dosing velocity from the storage vessel. A constant enzyme dosage level in the application is essential for many enzymes in order to obtain a homogenous performance level. In an ideal situation, the measurement of the enzymatic activity itself is preferred. However, such measurement often requires a time window of a few minutes or more, whereas on-line information is preferred for accurate enzyme dosage. This is an important issue since enzyme dosing may become essential for obtaining a homogenous performance level when the incubation time is more and more reduced.

On-line measurement of enzyme level may be done via an indirect method, i.e. by measuring the amount of protein via spectroscopy. This can be achieved by using a flow-cell unit and measuring the absorption at a wavelength of 280 nm. The aromatic tyrosine residue absorbs at this wavelength and since most proteins contain tyrosine residues, the wavelength can be used often. Where the enzyme applied does not contain this amino acid, measurement at approximately 275 nm (absorption by tryptophan) or 260 nm (absorption by phenylalanine) provides an alternative. A prerequisite for applying this method is that the measured absorption can be

correlated directly with the amount of active enzyme. This means: (i) the enzyme must be applied under conditions where it maintains its full activity (no significant inactivation may occur); (ii) no other ingredients in the application should absorb at the wavelength applied; (iii) the fabric should not release any chemical/component which absorbs at this wavelength; and (iv) the enzyme preparation used should be rather pure with respect to protein content. If this last item is not the case, enzyme monitoring by this method cannot be achieved, since the level of pick-up by the fabric of both enzyme and contaminant protein may be different. As a possible consequence, a shift in performance during process operation may be observed.

In a situation where there is no specific pick-up of enzyme by the fabric but only via adsorbing a film of enzyme-containing water, the enzyme dosage is relatively simple. Under circumstances where the enzyme maintains its full activity, it leaves the enzyme bath by adsorption of enzyme solution on the squeezed fabric. Maintaining the level of enzyme solution is enough in this situation to ensure constant enzyme dosage and thus performance.

When the enzyme incubation in a continuous process has to be executed in the enzyme bath itself, a comparable, but not identical, situation occurs to that in a batch process. In the batch situation, all fabric undergoes enzymatic action at the same time. The impact of a possible shift in enzymatic activity during incubation will be experienced by the total fabric batch. In a continuous operating process, fluctuations in the enzymatic activity will not be experienced by the total fabric batch. Therefore, the enzymatic activity has to be kept constant. This can be realised by applying conditions whereby no loss of any enzymatic activity will occur. In practice, some inactivation is frequently faced in spite of maintaining stable temperature and pH conditions. Therefore, monitoring the activity is essential at all times. In this situation, measurement of the enzymatic activity on a regular basis will be accurate enough, although on-line activity measurement is still preferred.

In any case, an enzyme bath cannot be used indefinitely. Inactivated and unfolded protein will accumulate in the bath and may influence the performance of subsequent processes (dyeing and finishing) by binding or deposition on the fabric after passing a certain threshold of inactivated protein concentration. To prevent such unwanted shift in product quality, it is recommended that each continuous process be started with a new, fresh made enzyme solution on a regular basis. How many hours/days such enzyme solution can be used depends on the enzyme itself and the set of parameters applied in the process, and cannot be predicted in advance. However, one should be aware that an unexpected shift in product quality (increasing inhomogeneity) might be triggered back to the freshness of the

enzyme solution used. Inactivation of the enzyme after application can be achieved by conducting the fabric through an alkaline solution followed by passage through a steamer.

5.4.2 Practical issues

Although at present, enzyme technology has been widely embraced in the textile area, the fine detail of the technology is not always known. Those employees who are working with enzymes on a daily basis, should be given practical training in how to handle enzymes safely, both for the sake of the person involved, as well as for enzyme product quality (enzymatic activity maintenance). Additionally, factory managers should also have practical knowledge of how to manage enzymes in order to create safe working facilities and environments for employees. In this section some enzyme handling situations will be discussed.

5.4.2.1 Enzyme storage

Enzymes which are delivered to the factory site, no matter in what form, have to be stored before they are used in an application. Enzymes will often, if not always, be delivered in closed plastic bags or drums, depending on the volume and type of formulation. During storage, high values of both temperature and humidity should be avoided. The environment in which the enzymes are stored should preferably be an isolated room within the factory which is kept cool, dry and well ventilated. Ventilation will remove any possible enzyme dust or aerosols generated on (un)packing enzyme products, and will secure a hygienic and safe environment for the employee. Upon storage of liquid formulated enzyme, temperatures below zero should be avoided at all times.

5.4.2.2 Enzyme preparation for application

Handling enzymes always has to be done in well-ventilated areas, no matter where in the factory. When opening a closed bag or drum containing solid enzyme product, handling procedures which generate high quantities of dust should be avoided. In the case of formulated enzyme product (granules), crushing the granules will generate dust and should also be avoided. In a situation handling enzyme powder, the transfer of enzyme powder into a different container (or whatever) should not be achieved by vigorous pouring but in a gentle way, for instance by ladling. A protective facemask, safety glasses and gloves should be used at all times during enzyme handling. After taking away the required amount of enzyme, the bag or

drum has to be closed immediately in order to minimise the amount of water uptake (protecting the remaining enzyme). In handling concentrated liquid enzyme formulations, the enzyme transfer should be done under hygienic conditions to avoid contamination by microbes. For the same reason, the number of times enzyme is taken from the same drum should be limited.

To prepare a diluted enzyme solution in water for the application, no matter if the concentrated enzyme product is in the liquid or solid form, the addition of enzyme to the water medium should be done as the last step in the preparation procedure. The order of addition will be such that first all other ingredients such as buffer, surfactants, and salts are introduced and dissolved in the medium. This medium should then be brought under the desired conditions of pH and temperature, and checked before the enzyme is dosed. If spillage occurs during this enzyme preparation, no matter if it is a concentrated or dilute enzyme solution, cleaning should be done directly before drying out can take place (prevention of dust formation).

5.4.2.3 pH maintenance during application

An enzyme is catalytically active in a certain pH range, which is different for each particular enzyme. Normally, at the start of the enzyme application, the pH is set by addition of acid, base or buffer of a particular strength. During application, the pH should be regularly monitored, preferably online. In a situation where the pH has shifted, correction may be required. pH shift may occur if an acid (most common in hydrolytic reactions) or base is produced in the catalytic reaction. Correction of the pH upon shift should not always be carried out. Each enzyme exhibits a certain pH tolerance: the enzyme is active for 80% or more of its maximal obtainable activity in a certain pH range. As long as the observed pH shift is within this range, no correction is required. To optimise the use of this tolerance range, the initial pH setting may be done not in the middle, but at one border of this range, creating a maximal possible pH range in which the pH can shift during ongoing enzymatic reaction without major impact on the enzyme activity.

If a pH correction during application is required, several methods are possible. Preferably, diluted acid or base (depending on the pH shift direction) is directly added to the application medium under vigorous stirring. In this way, the local high or low pH at the place where the acid/base is introduced into the application system will be kept as short as possible and thus prevent inactivation of the enzyme by pH-induction. Alternatively, an identical buffer solution with an increased strength can be added. The disadvantage of this method is enzyme dilution in the application. The method of tapping off a small volume of the incubation medium, and addition of

acid/base to this fraction prior to re-entering the application should be avoided since the enzyme in this fraction will be inactivated (the acid/base load needed to realise the required pH shift in the whole incubation system will result in an overcompensation of the pH value in this small fraction). This method can be used only in the case of immobilised enzyme systems, where the enzyme is drained off prior to acid/base addition.

In a follow up execution of this application, the use of a buffer system with increased strength is recommended once a required pH shift is experienced.

5.4.2.4 Enzyme inactivation at closure of the application

Often, irreversible inactivation of the enzyme at the end of the application is beneficial because ongoing enzymatic action may lead to undesired effects like extended tensile strength loss and 'overperformance' in the case of cellulase application. Inactivation of the enzyme by disruption of its three-dimensional structure can be easily realised by shifting the pH and temperature to extreme values for a relative short time period. Using normal, not thermostable enzymes in the application, a treatment for 5 to 10 minutes at a pH level above 10 and a temperature above 80°C is effective in destroying the enzymatic activity completely. Dealing with a thermostable enzyme, the treatment time and/or temperature should be increased. Shifting the pH to an extreme acidic value is also possible in principle. However, most applicable enzymes have the highest pH stability in the mild acidic range. Therefore, the biggest pH shift is realised when moving towards an extreme alkaline value. Additionally, cotton fabric is more resistant to extreme alkaline treatment in comparison to extreme acidic treatments. Other alternatives for enzyme inactivation, such as proteolysis, precipitation, chemical modification and protein unfolding inducer or enzyme inhibitor addition, are all less attractive for reasons of time, economic costs and/or efficiency level.

5.4.2.5 Compatibility additives

In enzyme applications, additives are regularly used for different objectives. Compatibility of those additives with the enzymatic activity must be checked prior to application because, especially in the groups of surface active agents and chelators, there are components which are not compatible. In the group of surface active agents, the charged components often have the ability to bind to oppositely charged amino acid side chain residues upon which an irreversible protein unfolding process is started-up. If the presence of a surface active agent is required, a non-ionic component is recommended.

In the situation where charged agents in the application are essential, no matter if these are anionic or cationic, the combination of such a charged agent and a non-ionic agent is recommended. Often a reduced negative impact of the charged surface active agent is observed upon application of such a mixture. One explanation for this observation is that the hydrophobic binding of the non-ionic agents to the surface of the enzyme prevents interaction with the charged agent. As an example, it is known from the literature that cellulases are sensitive to both anionic and cationic surfactants (Ueda *et al.*, 1994) whereas they are compatible with non-ionic surfactants. These non-ionic surfactants even improve the enzyme's performance upon addition in the application. After extensive study, Kaya *et al.* (1995) came to the conclusion that non-ionic surfactants hinder the binding of the enzyme on cellulose by reduction of the binding strength. In this way, the desorption rate of the enzyme is increased leading to an improved enzyme activity and performance level.

Also, for structural reasons, additives from chelators are potentially not compatible with enzymes containing a coordination ion. Each chelator has its own affinity towards a certain ion, which influences the strength of complexation. As a consequence, when the binding affinity towards an added chelator is higher than towards the protein, the ion will be extracted from the enzyme, disrupting the structure and inactivating the enzyme. If the presence of a chelator is required in the application, it needs to have a lower affinity for the enzyme-bound ion and yet still function in the application. As an example, the application of the BioPrep 3000L enzyme can be mentioned in the bioscouring process of cotton fabric. The enzyme contains a calcium ion to maintain its three-dimensional structure and, therefore, addition of a strong chelator such as EDTA (ethylenediaminetetra-acetic acid) will destroy its activity (Novozymes, 1999) whereas a mild chelator variant like STPP (sodium tripolyphosphate) is compatible with the enzyme (Lange, 1999).

5.4.2.6 Enzyme dosage

It is frequently thought that the amount of enzyme activity (dosage) and incubation time are exchangeable in a linear way in the application and that the more catalytic breakdown that has been realised, the better the performance obtained. For some enzymes in certain applications this may be valid, more or less. However, the opposite is true in many situations dealing with (insoluble) polymers as substrates, as is the case in the textile industry. Other operational conditions may be more important or essential than the actual level of enzymatic activity, and in these situations, increasing the incubation time, or raising the enzyme dosage will not result in an improved performance level. As an example, the application of cellulase on cotton

fabric to produce biopolished fabric should be mentioned. Performance will be achieved only upon passing a certain threshold value of enzymatic breakdown of the cellulose fibres. Removal of the enzyme-weakened microfibres is achieved by the application of shear force above a certain minimal level (Lenting and Warmoeskerken, 2001). Under this threshold value of shear force, no microfibre removal will be realised, no matter if the enzyme dosage and/or incubation time is increased. In a situation with a shear force level above the minimal value, a higher enzyme dosage will not lead, *a priori*, to performance in a shorter time frame. Increasing the enzyme dosage, will, besides a general increase of hydrolysis velocity, result in a different pattern of cellulose hydrolysis, but these modified kinetics do not lead, *a priori*, to accelerated performance. Incubation time and enzyme dosage have to be handled in this situation as being related, but not linearly exchangeable parameters.

When a concentrated liquid enzyme product is available for application where fabric is already present in the incubation medium, it is recommended that the enzyme solution in the incubation medium is diluted prior to addition to the fabric. This procedure can prevent local overperformance and/or inhomogeneous performance since effective mixing is often not realised at the time of enzyme addition (mixing device on hold during this handling).

In a situation where a rather concentrated enzyme solution has to be prepared from granulated enzyme product, it is recommended that the granulated enzyme is dissolved in a separate vessel, followed by a filtration procedure prior to introduction in the application. In this way introduction of possible insoluble substances originating from the granules can be avoided. Such insolubles may have some impact on the substrate, such as with inhomogeneous dyeing afterwards.

5.4.2.7 Repeated enzyme use

To make the enzyme technology more profitable, re-use of enzymes should be considered since they are relatively expensive. However, to ensure performance level during re-use, circumstances should be identical in all cycles of re-use. In a situation where processing agents other than an enzyme are used, which regularly occurs in the textile production area, one has to assure that accumulation of agents does not occur. A shift in the concentration of a processing agent, for instance a surface-active agent, may result in a shift in performance as a consequence. These agents should therefore be monitored both for underdose and overdose in the subsequent enzyme application cycles. Apart from its impact on performance, a shift in processing agent concentration may also have an effect on the stability of the enzyme and therefore on its half-life time.

On re-use of an enzyme in different subsequent cycles, one has to be alert for a different phenomenon: the potential impact of denaturated enzyme protein. During application, some enzyme will be inactivated and denaturated (protein unfolding). It may be possible that, after passing a threshold value of denatured protein, this protein becomes insoluble and is deposited on the fabric. If this phenomenon occurs, it may initiate problems later on in the dyeing and finishing processes. However, in the presence of a charged surface-active agent, the chance of such a redeposition process taking place is greatly reduced. If such an agent is not already present as a processing agent, it may be applied for this reason, if compatible with the enzyme.

To re-use an enzyme and avoid the above-mentioned potential problems, the isolation of the enzyme from the medium after each application cycle should be considered. This can be done in different ways in principle, but to be profitable it should be achieved in the simplest way possible, such as size-exclusion chromatography. Enzyme immobilised on a carrier can be isolated by simple drainage of the medium over a gauze filter.

5.4.2.8 Enzymatic activity

The supplier often guarantees the amount of active enzyme in an enzyme preparation by its activity. This enzyme activity is measured on a defined substrate using a fixed set of parameters. In many cases, the substrates used are easily soluble, modified substrates; for instance carboxymethyl- or hydroxyethylcellulose when measuring cellulase activity. Synthetic chromogenic substrates (like nitrophenyl cellobiosides for cellulases) are also used for convenience and reproducibility, and the method to be used is mostly available on request. Furthermore, different enzyme suppliers use different assay systems to measure the activity of the same enzyme type; for instance that of the cellulase enzyme. Enzymatic activity is expressed in Units (the amount of micromoles of product formed in one minute) per milligram of protein or product (volume). If a preparation contains pure enzyme, the activity measured per milligram of protein is called the specific activity. Each enzyme originating from a different (microorganism) source exhibits its own specific activity, but activities measured under different circumstances and using different substrates, not being the substrate in the application itself, cannot be directly compared with each other and cannot be linked directly to a certain performance level. Therefore, replacement of an enzyme by another of the same type but different origin in an application, maintaining all the parameters including enzyme dosage, as expressed in Units, does not, *a priori*, result in the same performance.

A given enzymatic activity for an enzyme preparation should be used only for quantification of the amount of active enzyme. For determi-

nation of the required dosage in the application, an activity–performance profile should be generated under the conditions of the real application. Likewise, a pH-activity profile, as given in the data sheet of an enzyme product, cannot be read as a pH–performance curve; such curve should be measured under the conditions of the application itself while monitoring performance instead of activity. The same is valid for the temperature–activity profile, especially when dealing with insoluble substrates. Upon binding of the enzyme to its substrate, the temperature stability may be improved. Often in the textile area, the substrates used are insoluble.

The last point for consideration is the fact that each different batch of the same enzyme product does not have an identical protein load *per se*, although the enzyme activity content may be the same. At first, a delivered enzyme product will contain a guaranteed minimal level of enzymatic activity. Since an enzyme undergoes some enzymatic inactivation upon storage between product generation and delivery, a certain overdose of enzymatic activity in the product will be used to guarantee a certain activity level. Secondly, each batch of enzyme produced will have a different history with respect to the processing efficiency and enzymatic inactivation profile during fermentation and downstream processing when the enzyme is (partially) purified.

5.4.2.9 Enzyme-containing wastewater

After enzymatic treatment, the medium drained off will contain protein, no matter whether the enzyme has been inactivated or not. Its presence in wastewater will lead to elevated levels of oxygen demand in municipal water purification units and often to increased costs of wastewater treatment if cost calculations are based on oxygen demand.

To limit the increase in wastewater treatment costs, the waste enzyme protein should be removed. This can be realised by various methods. The solubility of a protein in water is strongly reduced at its iso-electric point, the pH value at which the sum of all charged amino acids is zero. Shifting the pH of the wastewater to that pH value will initiate flocculation of some proteins. After complete precipitation, the protein can be separated by gauze filtration. Other enzyme proteins may be made insoluble by denaturation with temperature and/or pH shift. When dealing with enzymes that are not precipitated by one of these methods, the protein may be bound to resins prior to drainage. Separation of intact enzyme from the wastewater using membrane technology is another alternative, but will be more expensive and may be not economical. The separated protein itself may be used as a feed ingredient.

5.5 Health and safety issues

Large-scale application of enzymes started in the late 1960s when the first enzyme (protease) was incorporated in cleaning applications. In the textile production industry broad enzyme application was first used in the desizing process. Initially, companies working with enzymes underestimated their potency in relation to health. During this initial period of broad enzyme application, handling of enzyme (protease) preparations frequently resulted in skin irritation of employees. Employees came in direct contact with enzymes during production (fermentation), downstream processing and formulation of the enzyme product and in its application. Since then constant attention has been paid towards the risks to health when handling enzymes. This ongoing attention has resulted in, among others, application of formulation technology for enzyme preparations, improved industrial hygiene practices and procedures, improved design of production and manufacturing plant and dust control equipment, and increased understanding of enzyme-induced sensitisation and health effects. For the detergent industry it can be stated that, as a result of all the above-mentioned improvements and strict maintenance of hygiene rules, detergents containing enzymes have been produced safely during the last 25 years without the problems faced in the starting period (The Soap and Detergent Association, 1995). Nevertheless, when the strict hygiene rules are not obeyed in practice, adverse effects cannot be excluded. As an example in the textile industry, an employee of a dye house developed cellulase-induced occupational asthma as a result of daily exposure to this enzyme in powder form for almost two years (Kim *et al.*, 1999).

5.5.1 Exposure routes and health risks

Exposure to enzymes may cause irritation and/or allergies either via inhalation or by skin and eye contact. These allergies are no different to other irritants like pollen and house dust. The human body recognises an enzyme as human-different material and starts production of allergic antibodies when enough material enters the body via inhalation of enzyme-containing aerosol. When these allergic antibodies are detected, the person in question is said to be sensitised and the presence of antibodies is an indication that the person has inhaled human-different material. Determination of these antibodies during regular medical control can be used as proof that enzyme material has been inhaled. Prolonged uptake of enzyme material via inhalation can lead to symptoms such as watery eyes, runny nose, scratchy throat and occupational asthma, as mentioned before. When the intake of enzyme material ceases, these symptoms should disappear. However, when the

doses of enzyme exposure are extremely high, this may cause irritation of the respiratory tract, resulting in symptoms such as congestion, difficult breathing and sore throat. However, under normal circumstances, with adequate controls or rapid action at the first signs of enzyme exposure, such situations should not occur.

Enzyme–skin contact will not lead to a sensitised human body. Skin irritation may occur in a situation of exposure to high levels of, especially, proteases, since the human body is relatively sensitive to this type of enzyme. The irritation caused by proteases is characterised by a weeping, red glistening appearance on the skin surface, which can be painful. The use of gloves and protective coveralls when working with large amounts of enzymes, no matter whether in granular or dissolved form, will prevent skin–enzyme contact. When small quantities of enzyme come into contact with the skin, a normal washing procedure with excess water should be effective in avoiding skin irritation.

Enzyme contact in the eye may have the same effect as skin contact: irritation. Rinsing thoroughly with excess water will minimise the effect of enzyme contact. The use of protective glasses and/or face shields is recommended when working with enzymes.

5.5.2 First aid

In a situation where all safety precautions are routinely followed, no contact with the active component of an enzyme product will occur. However, an accident with enzyme contamination as a consequence can occur at any time. Since the enzyme is soluble in water, water should be always used for the complete removal of the contamination upon direct exposure. It is therefore highly recommended to have safety showers and eye wash stations available in every area where possible enzyme contamination can occur.

For the various enzyme exposures the following actions are recommended:

- Ingestion: rinse the mouth and throat with tap water (the acid environment of the stomach inactivates the enzyme);
- Skin contact: wash the skin with plenty of water; remove clothing in case of contamination;
- Eye contact: rinse the eyes thoroughly with water for a long time (more than 10 minutes);
- Inhalation: remove the employee from the exposure area; in the event that the symptoms of irritation or sensitisation remain (shortness of breath and coughing), call or transport the person to a doctor.

5.5.3 Product design and allergenic potential

Enzymes are proteins which can catalyse certain chemical reactions. External proteins not belonging to the human body may have epitopes on their surface (local three-dimensional surface structures constructed by a combination of certain amino acids) which are recognised by antibodies when entering the human body. Every enzyme which is commercially exploited has to be previously tested in a series of toxicity tests (including determination of the doses, expressed as g/kg body weight, that are lethal for rats in 50% of the cases) in order to clarify its human and environmental toxicity potential. This information is incorporated in the MSDS (Material Safety Data Sheet) which has to accompany each enzyme product delivery.

The primary route for enzyme exposure which can potentially lead to allergy is inhalation. Therefore, the formation of respirable aerosols should be prevented as far as possible. The physical form of the enzyme can greatly influence the potential for such aerosol formation. Enzyme aerosols may be in the form of liquid droplets, mists, solid particles or dusts. Powdered enzyme products give the largest risk of exposure since they are easily aerosolised. For over 25 years, enzyme manufactures have formulated their products using granulation technology in order to prevent enzyme aerosol formation and the granulated products now available have excellent low-dust and abrasion resistance. The enzyme is encapsulated in these granules and in this way its release into the air is prevented. However, care must be taken not to crush them as the enzyme powder inside could then be easily aerosolised.

Enzyme formulations are also available in a slurry or liquid form. Slurries are more viscous than liquids and both have a minimum potential for aerosol formation, although situations have to be avoided where the chance for aerosol formation is increased. Examples of such situations are high pressure cleaning with steam, air or water and when any type of mechanical agitation is applied to the enzyme-containing liquid. Additionally, in the case of liquid enzyme spills, enzyme dust generation from dried material has to be avoided by washing away the enzyme material with water (Enzyme Technical Association, 1995).

A relatively new technology to reduce the allergic potential of enzymes is the modification of allergic epitopes on the surface of active and correctly folded enzymes by protein engineering. By use of this promising technology, variants of a protease from *Bacillus lentus* have been created with reduced allergenicity. Reductions to one-fifth of the level induced by the original protease have been measured by determination of the amount of allergy-specific antibody (Novozymes, 2001). Indications are that further modifications can reduce the allergenicity to almost zero without introduction of novel allergic epitopes.

5.5.4 Precaution and protection issues

An enzyme batch delivery should be accompanied by an MSDS. This contains information on how to handle the enzyme preparation and what safety precautions are required to protect the employee. Both employee and supervisor should have access to the sheets at all times. Additionally, enzyme manufacturers have manuals and booklets available for enzyme users, which will guide them in handling their enzyme products in a safe way. In the manual from Novozymes for instance, all safety, protection, monitoring and medical aspects are discussed (Novozymes, 2001). Also the Association of Manufacturers of Fermentation Enzyme Products (AMFEP), a European industry association founded in 1977, has produced an information guide concerning the handling of microbial enzyme preparations (AMFEP, 1994).

Proper work practice procedures are important in controlling enzyme aerosols and these have to be used in conjunction with engineering controls and personal protection equipment. These procedures include appropriate management systems for maintaining the necessary high hygiene standards, clear responsibilities for daily management, and availability of accurate trained personnel in the fields of medicine, industrial hygiene and engineering. The process plant should be engineered in such a way that risk of potential enzyme aerosol contact is minimised. Good local exhaust ventilation of the plant and enclosures are most effective instruments and should be preferably used at locations where enzymes are added into the process, at material transfer points and in the packaging rooms (Enzyme Technical Association, 2000). The process equipment should be a closed system when possible. Proper work practice procedures also include accessibility of washing facilities, cleaning equipment and disposal containers. Enzyme-containing spills and machinery should be cleaned directly by means of a vacuum system equipped with a high efficiency particulate air (HEPA) filter. Cleaning has to be done without brushing and/or sweeping since this will generate aerosol. The use of high-pressure water, air or steam has to be avoided for the same reason.

Employees who are working with enzymes should have access to personal protective equipment to prevent enzyme contact. This should protect the employee in a situation where process design and work-practice procedures are not appropriate. In other situations, such as clean up of spills, it will be the primary control method. Respiratory protection is used when airborne enzyme cannot be sufficiently controlled to a safe level. There are three main classes of respirators available: air purifying, air supplied and self-contained breathing equipment. The first class is most common in, for example, detergent facilities. The air-purifying respirator uses a cartridge to remove contaminants from the air. For enzyme-containing aerosols, a

HEPA filter cartridge is recommended. A higher level of respiratory protection is realised when using the air supplied system. In this situation, clean air is supplied to the wearer via a supply host. The self-contained breathing equipment obtains the highest level of protection and greatest freedom of movement and is, therefore, used in emergency situations. Once again, in normal operating conditions in a well-ventilated plant, the routine wearing of respirators is not necessary.

As noted before, skin and eye contact with enzymes should be avoided in order to prevent irritation. If there is a potential for eye or skin contact, protective wear and clothing should be used. Effective protective equipment include glasses, face shields, gloves and coveralls, and their use should be limited to the working area only and laundered after use.

5.5.5 Air monitoring

A continuously-operating air monitoring programme should be established in order to evaluate or screen the potential for employee exposure to airborne enzymes. Air samples should not only be taken at places where the highest risk of aerosol formation occur, but also in low risk places to get a complete overview of airborne dust existence within the plant. Historically, high volume air sampling was necessary because of the low sensitivity of the existing evaluation methods. Nowadays, operational evaluating methods are able to detect lower quantities of enzyme, which allow smaller samples for analysis. The benefits of working with small volume samples are that the required pumps are easier to calibrate, personal samples can be collected, and that the pumps are lighter, smaller and more mobile and do not require an external energy source. Upon sampling, airborne enzyme is collected on filters and the amount of enzyme is assessed either by measurement of the enzymatic activity or by ELISA (enzyme linked immuno sorbent assay). The sensitivity of the analytical ELISA method is high, while that of the activity measurement is dependent on which enzyme activity has to be measured (variable sensitivity). On the other hand, the analysis time for activity measurement is relatively short (less than one hour) when compared to that of the ELISA test (up to 12 hours). Which method is preferred will depend on the level of airborne enzyme expected and the time in which an analysis outcome has to be available.

The existence of enzyme exposure guides is important in a monitoring programme. They provide references for exposure levels that do not adversely affect the health of employees in an industrial environment where enzymes are used. As an example of a guide level, the ACGIH (American Conference of Governmental Industrial Hygienists) has established a threshold upper limit value of 60 ng/m^3 air for the protease enzyme subtilisin (The Soap and Detergent Association, 1995). This value is a ceiling

value and the industry strives to have airborne enzyme levels which are normally an order of magnitude below this ceiling value.

5.5.6 Medical monitoring

Apart from monitoring the airborne enzyme throughout the production plant, the employees of the plant should also be monitored for possible enzyme contact. An ideal situation is when an employee, before he or she starts to work with enzymes, is examined medically. Specific topics in this pre-examination are their history of allergies, asthma, eczema, smoking, previous chest diseases, acquisition of a pulmonary function and enzyme sensitivity baseline and an overview of common allergens present (skin prick test) to determine the atopy of the individual. During his or her working life with enzymes, the employee should be routinely monitored annually. Deviation from this scheme may be considered in situations where, as an example, a new enzyme is introduced or process modifications have been adapted. Items that should be reassessed are the lung (pulmonary) function and enzyme sensitivity. Additionally, smoking and any relevant history of illness symptoms should be evaluated since the previous medical examination. Finally, employees should inform management and medical experts of a situation of any respiratory problem upon which prompt medical evaluation can occur and sources of airborne enzyme exposure can be removed.

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