Enzyme Technology

For thousands of years natural enzymes made by microorganisms have been used to make products such as cheese, bread, wine, and beer. Enzymes are now used in a wide range of industrial processes. The study of industrial enzymes and their uses is called **enzyme technology.**

The advantages and disadvantages of using enzymes are **directly related to their properties:**

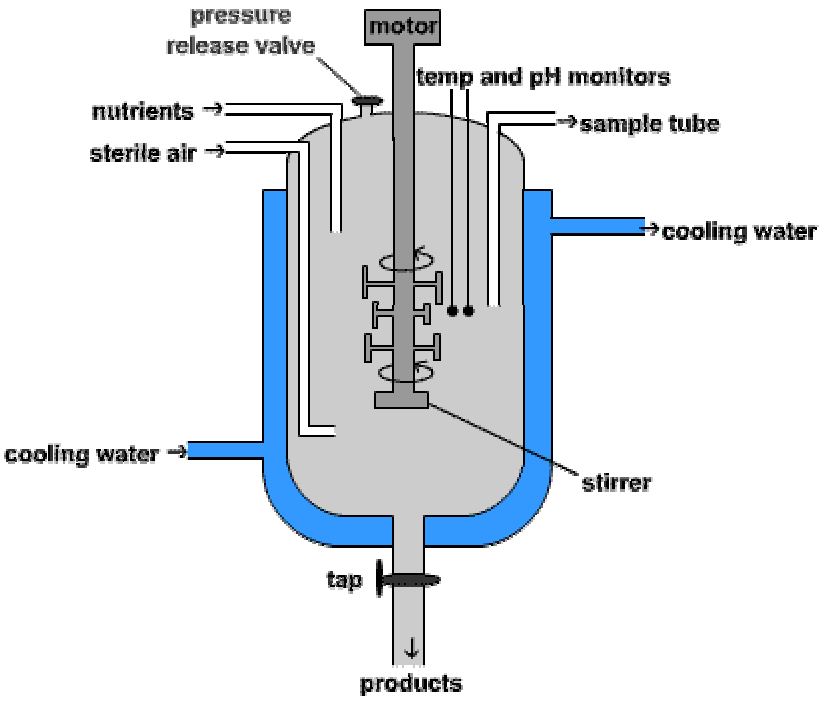
Advantages	Disadvantages
They are **specific in their action** and are therefore **less likely to produce unwanted by-products**	They are **highly sensitive to changes in physical and chemical conditions** surrounding them.
They are **biodegradable** and therefore **cause less environmental pollution**	They are **easily denatured** by even a small increase in temperature and are **highly susceptible to poisons and changes in pH**. Therefore the **conditions in which they work must be tightly controlled**.
They **work in mild conditions**, i.e. low temperatures, neutral pH and normal atmospheric pressure, and therefore **are energy saving**	The enzyme substrate mixture **must be uncontaminated with other substances** that might affect the reaction.

Microbes are still the most common source of industrial enzymes. Microorganisms produce enzymes inside their cells (**intracellular** enzymes) and may also secrete enzymes for action outside the cell (**extracellular** enzymes). The microorganisms selected are usually cultured in large fermentation chambers (*known as **fermenters** – see later*) under controlled conditions to maximise enzyme production. The microorganisms may have specific genes introduced into their DNA through ***genetic engineering***, so that they produce enzymes naturally made by other organisms - this is explained in further detail under the genetic engineering section of this unit.

Growing microbes in a fermenter

Given a suitable nutrient medium and the right conditions (temperature, pH, oxygen levels (many microbes are *obligate anaerobes,* i.e. are killed by oxygen), it is easy to grow microbes on a laboratory scale in Petri dishes, test tubes and flasks. However, producing substances such as penicillin from microbes on an industrial scale causes serious problems because massive numbers of organisms have to be grown for commercial use.

The microorganisms are grown in very large vessels called **fermenters** – as shown in this simplified diagram:



The large stainless steel cavity is filled with a sterile nutrient solution, which is then **inoculated** with a pure culture of the carefully selected fungus or bacterium. Paddles rotate the mixture so that the suspension is mixed well. As the nutrients are used up, more can be added. Probes monitor the mixture and changes in pH, oxygen concentration and temperature are all computer controlled. A water jacket surrounding the fermenter contains fast flowing cold water to cool the fermenter since fermentation is a heat generating process. Most of the air, including carbon dioxide and other gases produced by cell metabolism, leave the fermenter by an exhaust pipe.

Requirements for the production of microbes in fermenters:

- **Oxygen** is needed for aerobic respiration of (some) micro-organisms – others are strict anaerobes and oxygen must be excluded
- a source of **Carbohydrate** is needed as an energy source for respiration to release energy needed for growth.
- a source of **Nitrogen** is needed need nitrogen for protein synthesis – **Ammonia** (NH_3) and **urea (**($NH_2)_2CO$) are both widely used as (cheap) sources of useable nitrogen

Isolating the Enzyme

Pure enzymes are needed for commercial use; therefore microbes must be grown in **aseptic conditions**, free from contaminants - such as unwanted chemicals - and other microbes. It is necessary to prevent contamination with other bacteria since:

- there may be competition for nutrients;
- the required enzyme may not be produced as readily;
- the end-product may be contaminated and unsafe.

The required enzyme that is finally produced must also be isolated from the microbial cells.

- **Extracellular** enzymes are present in the culture outside the microbial cells, since they have been secreted. They are often soluble in water, so they can readily be extracted from the culture medium and purified. Less common in Nature (though genetic engineering can be used to modify cells to promote this), these enzymes are cheaper to produce and tend to be more stable – they are therefore the preferred choice, **when available!**
- To obtain an **intracellular** enzyme, the microbe cells are harvested *(by filtration or centrifugation)* from the culture and are then broken up. The mixture is next centrifuged to remove large cell fragments and the enzymes (**all** of them!) are precipitated from solution by a salt or alcohol. The **required** enzyme must then be purified by techniques such as electrophoresis or column chromatography.

This last process is complicated and expensive, so these enzymes are only used when no other alternative is available. By their very nature, they tend to be more sensitive to their operating conditions, which makes their commercial use less easy. On the other hand, they are much more common in Nature!

Table comparing intra- and extra-cellular enzymes:

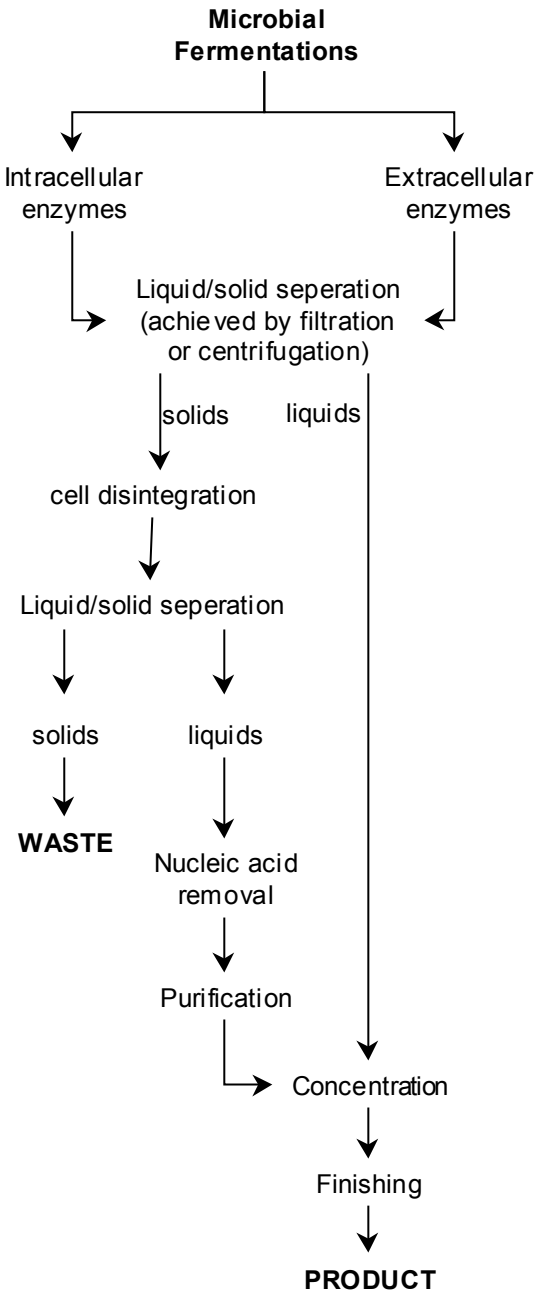
Intracellular enzymes	Extracellular enzymes
More difficult to isolate	Easier to isolate
Cells have to be broken apart to release them	No need to break cells – secreted in large amounts into medium surrounding cells
Have to be separated out from cell debris and a mixture of many enzymes and other chemicals	Often secreted on their own or with a few other enzymes
Often stable only in environment inside intact cell	More stable
Purification/downstreaming processing is difficult/expensive	Purification/downstreaming processing is easier/cheaper

Microorganisms such as bacteria and fungi are **saprobionts** i.e. they feed **saprophytically,** secreting enzymes onto their food – making them a good source of extracellular enzymes. For example, the fungus *Aspergillus niger* produces an enzyme called ***pectinase,*** which breaks down pectin, a substance found in the cell walls of plant cells. The fruit juice industry uses pectin widely, since when fruit is crushed to extract the juice, pectin prevents some being released and also makes the juice cloudy.

The stainless steel fermenter with complicated control systems is not actually the most expensive part of the process. Almost 80% of the cost is accounted for by **downstream processing:** the isolation, extraction, and purification of the product at the end of the culture in the fermenter.

Downstreaming uses a variety of techniques. In the first stage cells need to be separated from the liquid part of the suspension. This can be done by sedimentation, centrifugation or filtration. If the cells themselves are the desired product (e.g. for single-cell protein production for animal feed) then they need to be sterilised, washed, dried and packaged. If the desired product is a chemical within the cells, the cells have to be broken apart to release the chemical and the cellular components removed. The desired chemical is then extracted and purified by a number of techniques such as precipitation and chromatography.

Finally, the purified chemical has to be dried and packaged in a suitable form. In the case of the enzymes in biological washing powders, this means coating the granules with wax to ensure that they remain dry until used – otherwise the enzymes would digest themselves!



Medical uses of enzymes

Diagnostic:

Reagent strips have been designed to perform rapid and semi-quantitative analysis for glucose. They are easy to use and require no addition laboratory equipment or reagents. A **Clinistix** contains molecules of two enzymes fixed onto the end of a plastic strip. When this is dipped into a sample, the first, **glucose oxidase,** converts any glucose molecules, by reaction with atmospheric oxygen, into gluconic acid and hydrogen peroxide. The second enzyme, **peroxidase,** then enables the hydrogen peroxide to react with an indicator to give a purple colour. A colour chart on the strip will match the shade of purple to the glucose concentration. The idea of fixing an enzyme to a plastic support is the basic principle of **biosensors** - mobile, cheap and accurate sensors which can monitor a number of biochemicals in blood, urine and also in food and soil. Over the next few years, the use of biosensors is likely to increase dramatically.

Step 1:

Glucose oxidase

Glucose ----------> Gluconic Acid + Hydrogen Peroxide

Step 2:

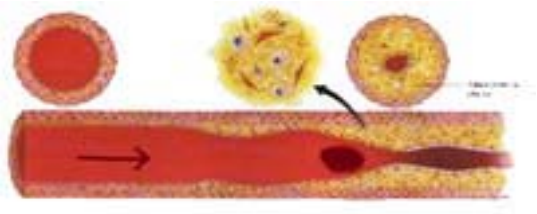
Peroxidase

Hydrogen peroxide + (reduced) orthotolidine ----------> Water + (oxidized) orhotolidine

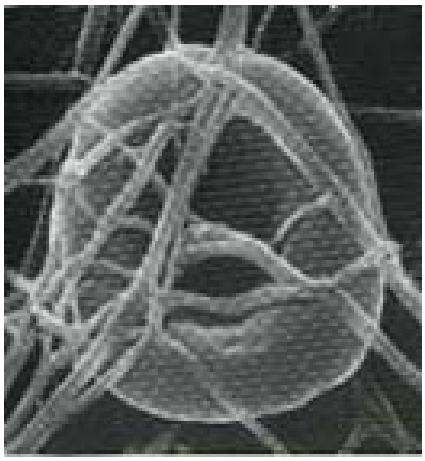
Brown *Blue*

Treatment:

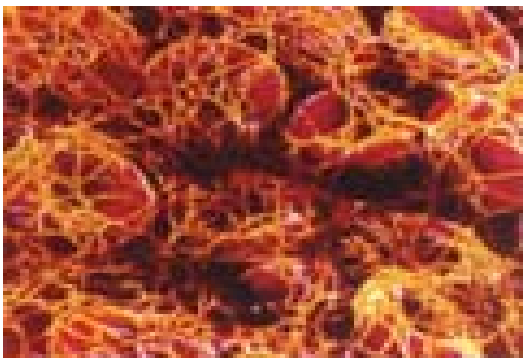
The approved treatments for strokes are i.v. prescription drugs Urokinase, Streptokinase and t-PA (tissue-plasminogen-activator). All three are available in the form of intravenous infusion only. To work best, they must be given **within three hours from the onset of the attack**.



A floating time bomb, an embolism could be trapped and block any artery.



Fibrin filaments wrap around and entrap a single red blood cell.



The beginning of a blood clot: Platelet and Red Blood Cells are trapped in a network of fibrin cables.

The industrial use of enzymes (using the whole microbe)

Historically, three examples of the industrial use of microbes (and their enzymes) are:

Brewing:

In which yeast (*Saccharomyces cerevisiae)* reacts with the sugars in fruit or malted barley to produce ethanol and carbon dioxide. In Nature, the yeast is competing with bacteria for the available sugar in the wild fruit. Its response is a form of 'chemical warfare', since the ethanol it makes it poisonous to many bacteria and, indeed, ethanol can be used as a disinfectant (though it stings a lot!). The process of fermentation takes several days or weeks and results in a product with a maximum alcohol content of about 12% - above which the yeast is itself killed. More alcoholic beverages can only be made by distilling the raw brew. From wine we get brandy, cider gives Calvados, ale gives whisky.

Surplus yeast could then be used to mix with flour and water to make (leavened) bread. Hence brewing and baking are closely related. In baking, the carbon dioxide is the important product, since it makes the dough rise. the ethanol evaporates off in the baking, so you cannot get drunk by eating bread! The reason why 'in-store bakeries' are so popular in supermarkets is that the smell of baking bread (and the ethanol) in the air circulates throughout the store and this stimulates our 'hunger centre' and so we buy more - quite true!

These days the surplus yeast is heated and processed to make Marmite.

Vinegar production:

Louis Pasteur was employed by Emperor Napoleon III in 1864 to research why (sometimes) wine went 'off' or turned to vinegar. Pasteur soon showed that the historic 'spontaneous generation' theory was wrong – substances did not spontaneously go 'bad'; instead he formulated the modern 'germ theory'. This states that it is the existence of microbes which makes food rot. The secret to keeping wine was thus to keep the microbes out i.e. bottle it, rather than storing it in open casks. To make vinegar, wine is slowly pored over oak chips in a tall tower, open to the air. Bacteria (*Acetobacter)* on the wood oxidise the ethanol in the wine and turn it into ethanoic acid or vinegar, giving out a great deal of heat as well. If the vinegar is made from fermented raisins and stored in oak vats (similar to the *solera system* used for making sherry) then the sweet, highly-prized ***Balsamic vinegar*** is made – mainly around Modena in Italy. Note how different forms of 'sweet and sour' dishes are a part of local cuisine from all over the World!

Yoghurt production:

Milk goes sour within a few hours in the hot conditions common in the Middle East. If stored in a leather bag and mixed with a suitable starter culture, however, it rapidly turns into yoghurt, which will keep for several days. This happy accident led to the development of the modern industry, which thus has its roots in Biblical times (Abraham was said owe his longevity to drinking yoghurt). In Russia, *Kefir* is a similar ancient product with a fascinating modern commercial history, beginning in 1908 with the attempted seduction of a Prince Barcharov, the kidnapping of a beautiful maiden (Irina) and a court case with a fine of 'the Prophet's Grains', which were the sacred starter culture for Kefir. In 1973 the Minister for Food in the USSR wrote to Irena thanking her for bringing Kefir to the Soviet people!

The industrial use of enzymes (not using the whole microbe)

Leather:

The earliest example of enzymes in industry is a colourful one! To make leather soft, it has to be **bated,** which means that some of the protein fibres are removed. Otherwise, the leather will be hard - perfect for the soles of shoes but of little use for anything else.

The Roman writer Pliny reported the use of pigeon droppings for this process over 2000 years ago. Later, leather was bated by smearing it with dog excrement! People used to go around the streets collecting dog turds and then rubbed them into the skins by hand, paddle or by trampling it in by foot.

By the early 1900's it was known that the excrement was rich in bacteria which produced p**roteases**, which degraded part of the leather. It was a highly skilled job to prevent the enzymes damaging the leather, which is largely made up of protein. But thanks to the The German scientist Ršhm, developed a standardized ***bate*** in 1908, based on an extract from the pancreases of slaughtered animals. This contained trypsin - one of a mixture of enzymes found in the digestive system. Since then, all bates have been based on enzyme preparations, though now bacterial and fungal enzymes are used instead.

Washing powders:

Ršhm was quite a genius - he was the first to examine the chemical composition of dirt on laundry and he came up with the idea of using the pancreatic extract to wash clothes. His wife tested trypsin at home on their dirty underwear - and found it was excellent! When soaked overnight, their clothes became clean and the water became dirty. So, he patented his idea and in 1914, developed the first enzymatic washing agent. It was so effective that only a small quantity was required: it was sold as a spot remover. Unfortunately German housewives were used to bulky washing powders that produced lots of lather so they regarded it with suspicion. In 1915, some people even thought it was a hoax. The product was investigated by scientists who found that it really did work – indeed, it was about 50 years ahead of its time: it wasn't until the 1960s that enzymatic detergents gained widespread acceptance.

The mass-production of an alkaline protease suitable for wash conditions began in 1962. Unlike trypsin, this wasn't an animal extract but a product of microbial fermentation. This new enzyme was initially shunned by detergent manufacturers but there were exceptions. In 1963, it was incorporated into ***Bio-tex,*** which took the market by storm. Industry began to take notice of enzymes and by 1967 their widespread use in domestic detergents was commonplace.

Enzymes used:

These are produced from *Bacillus licheniformis*. They are usable at high pH and temperatures up to 60^{o} C and are all relatively non-specific proteases. They attack the C-terminal of carboxyl amino acids producing small peptides which can be readily dissolved by the detergent. There is currently considerable interest in developing better proteases for washing powders through protein engineering, particularly in engineering oxidation-resistance into the proteases.

Engineered Subtilisin for improved wash performance

Not just proteases

Since the 1990's, amylases have also been added to detergents to remove stains from spaghetti, sauces, oatmeal and baby foods. In 1988 the first detergent lipase was released - the first commercial enzyme to be produced from a genetically-modified organism (GMO). Today more than 90% of detergent enzymes are made from GMOs.

The detergent industry has been the largest market for industrial enzymes for over 25 years, accounting for 37% of world sales of enzymes. Apart from laundry detergents, many automatic dishwashing detergents now also contain enzymes.

To maximise the effectiveness and to be as economical as possible in the production process the enzyme molecules must be brought into maximum contact with the substrate molecules. This can be achieved by mixing the solutions of enzyme and substrate in suitable concentrations. **However t**his means that the enzyme is 'lost' with each batch of product made and that the end-product will be contaminated too – as in cheese manufacture:

Cheese making: Warm milk is mixed (about 2000:1) with the enzyme **rennin (***rennet)* (formerly extracted from (dead) calves' stomachs, but now produced from bacteria) and allowed to react for several hours. The ***caesinogen*** in the milk is uncoiled and clots to ***casein***. This turns the milk solid. The **curds** (solid) are then cut with a knife and the **whey** (liquid) drained away and fed to animals (remember Miss Tuffet?). The chopped up curd is then salted and placed in a mould before squeezing to remove any trapped air (a process known as 'cheddaring' – hence Cheddar cheese). Sometimes the cheese is then dipped in brine or a solution of fungal spores to inoculate it and produce a surface 'rind'.

The cheese is then left at a constant, low, temperature (in the old days, in a cave, hence many cheeses are associated with cave-rich districts) to mature. this may take up to a year or more, so cheese-making was an important way of preserving a valuable food through the winter in the days before refrigeration.

The 'blue' in cheeses such as Stilton, is added by pushing spore-covered wires (*Penicillium notatum*- the same fungus that gave us penicillin) into the partially ripened cheese. This fungus needs oxygen to make the blue pigment, so holes have to be made in the cheese – the more holes, the faster the blue veins develop.

The role of the rennin in young mammals is to clot the mother's milk in baby's stomach. This then 'tricks' the stomach into keeping the contents there for several hours, thus allowing protein digestion and the

mother to get some (much-needed) rest! In most mammals the rennin is only made until the animal is weaned, but in Caucasian people, milk was (historically) drunk throughout life and so the enzyme continues to be made, even in adults. Also made is the enzyme **lactase**, which breaks down the milk sugar and stops the bacteria from fermenting it in the colon, with subsequent large volumes of gas produced and embarrassing side–effects!

Producing a pure product more cheaply – re-using the enzyme

It would obviously be more economic to retain the enzyme and so be able to re-use it for several batches of product. In addition, the product would be pure and uncontaminated by any enzyme (though, since this is a natural product and a simple protein, it is unlikely to do any harm to the end-user). However, the enzyme needs to be in contact with the substrate in order to react.

The solution to this dilemma is to use '**immobilised enzymes':**

- By attaching the enzyme molecules to an inert surface (such as plastic beads) and then bringing the surface into contact with a solution of the substrate. This method has the advantage of enabling the enzyme molecules to be used over and over again, with the result that a lot of product can be made from a relatively small amount of enzyme.

An example of continuous production using an immobilised enzyme is:

- Fructose syrup production from glucose using ***glucose isomerase***

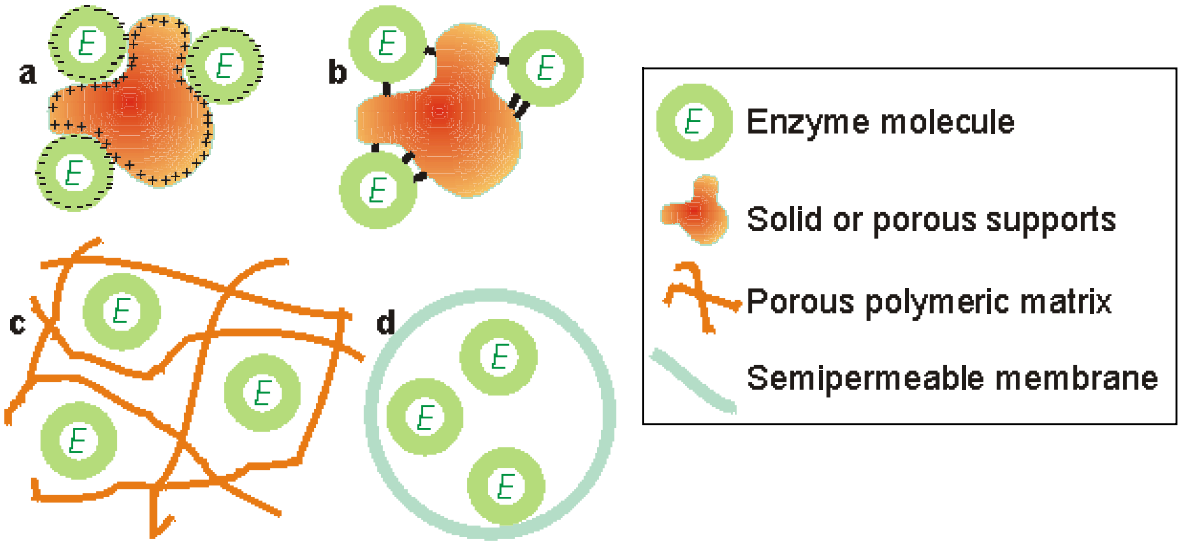
Improving the Enzyme: Immobilsation

As enzymes are catalytic molecules they are not directly used up by the process in which they are used. However due to denaturation, they do loose activity with time. Therefore they should be stabilised against denaturation. When the enzymes are used in a soluble form they can contaminate the product, and its removal may involve extra purification costs. In order to eliminate wastage and improve productivity the enzyme and product can be separated during the reaction. The enzyme can be imprisoned allowing it to be reused but also preventing contamination of the product – this is known as **immobilisation**.

Unstable enzymes may be **immobilised** by being attached to or located within an insoluble support, therefore the enzyme is not free in solution. Once attached, an enzyme's stability is increased, possibly because its ability to change shape is reduced.

There are four main methods available for immobilising enzymes:

a. Adsorption in glass or alginate beads – enzyme is attached to the outside of an inert material
b. Cross-linkage to another chemical e.g. cellulose or glyceraldehydes.
c. Entrapment in a silica gel – enzyme is held in a mesh or capsule of an inert material.
d. Membrane confinement



Compared with free enzymes, immobilised enzymes have several other advantages:

Advantages of immobilisation	Disadvantages of immobilisation
1. Easier to separate enzyme and products	1. Immobilisation may alter shape of enzyme
2. Allows catalysis in unfavourable media	2. May alter catalytic ability
3. Increases stability and can be manipulated easily	3. Enzyme may become detached
4. Allows continuous production/enzyme used for longer	4. Expensive
5. Enzyme can be recovered and reused	
6. Enzyme does not contaminate product/no purification required	

Improving the Enzyme: Stability

The **stability** of an enzyme refers to its ability to retain its tertiary structure so that it continues to be effective under a wide range of conditions. Most enzymes are **relatively unstable** and work **only within narrow ranges of temperature** and **pH**. They quickly become denatured when subjected to unnatural environments. Many industrial processes require enzymes to work in the presence of chemicals such as organic solvents, at high temperatures and extremes of pH – conditions with cause most enzymes to lose their shape and become inactive. It is possible to overcome this problem by taking advantage of microbes that live naturally in harsh environments.

Organisms evolve to produce enzymes that are adapted to their environmental conditions. Some bacteria are adapted to extreme environments e.g. some bacteria may live in hot volcanic springs. They will produce thermostable enzymes that do not denature at high temperatures – they work effectively in the temperature range 65-75°C. They will also remain effect at lower temperatures for much longer than 'normal' enzymes and so are the preferred choice for industry. These enzymes are also resistant to organic solvents and tolerate a wide range of pH. The gene for a thermostable enzyme can be isolated from the natural bacteria and transferred to a microbe 'host' that can be used in the industrial process. This enables a thermostable version of the desired enzyme to be produced.