**CHROMATOGRAPHY**

In many fermentation processes, chromatographic techniques are used to isolate and purify relatively low concentrations of metabolic products. In this context, chromatography will be concerned with the passage and separation of different solutes as liquid is passed through a column, i.e. liquid chromatography. Chromatographic techniques are also used in the final stages of purification of a number of products.

Depending on the mechanism by which the solutes may be differentially held in a column, the techniques can be grouped as follows:

(a) Adsorption chromatography.

(b) Ion-exchange chromatography.

(c) Gel permeation chromatography.

(d) Affinity chromatography.

(e) Reverse phase chromatography.

(f) High performance liquid chromatography

**ADSORPTION CHOMATOGRAPHY**

Adsorption chromatography involves binding of the solute to the solid phase primarily by weak Van de Waals forces.

The materials used for this purpose to pack columns include inorganic adsorbants (active carbon, aluminium oxide, aluminium hydroxide, magnesium oxide, silica gel) and organic macro-porous resins.

Di-hydro-streptomycin can be extracted from filtrates using activated charcoal columns. It is then eluted with methanolic hydrochloric acid and purified in further stage

Active carbon may be used to remove pigments to clarify broths. Penicillin-containing solvents may be treated with 0.25 to 0.5% active carbon to remove pigments and other impurities

Macro-porous adsorbants have also been tested. The first synthetic organic macro-porous adsorbants. These resins have surface polarities which vary from non-polar to highly polar and do not possess any ionic functional groups.

**ION EXCHANGE CHOMATOGRAPHY**

Ion exchange can be defined as the reversible exchange of ions between a liquid phase and a solid phase (ion-exchange resin) which is not accompanied by any radical change in the solid structure.

Cationic ion-exchange resins normally contain a sulphonic acid, carboxylic acid or phosphonic acid active group. Carboxy-methyl cellulose is a common cation exchange resin. Positively charged solutes (e.g. certain proteins) will bind to the resin, the strength of attachment depending on the net charge of the solute at the pH of the column feed. After deposition solutes are sequentially washed off by the passage of buffers of increasing ionic strength or pH.

Anionic ion-exchange resins normally contain a secondary amine, quaternary quaternary amine or quaternary ammonium active group. A common anion exchange resin, DEAE (diethylaminoethyl) cellulose is used in a similar manner to that described above for the separation of negatively charged solutes.

Other functional groups may also be attached to the resin skeleton to provide more selective behavior similar to that of affinity chromatography.

The appropriate resin for a particular purpose will depend on various factors such as bead size, pore size, diffusion rate, resin capacity, range of reactive groups and the life of the resin before replacement is necessary.

Weak-acid cation ion-exchange resins can be used in the isolation and purification of streptomycin, neomycin and similar antibiotics.

In the recovery of streptomycin, the harvested filtrate is fed on to a column of a weak-acid cationic resin such as Amberlite IRC 50 which is in the sodium form. The streptomycin is adsorbed on to the column and the sodium ions are displaced.

Flow rates of between 10 and 30 bed volumes per hour have been used. The resin bed is now rinsed with water and eluted with dilute hydrochloric acid to release the bound streptomycin.

A slow flow is used to ensure the highest recovery of streptomycin using the smallest volume of eluant. In one step the antibiotic has been both purified and concentrated, maybe more than 100-fold. The resin column is regenerated to the sodium form by passing an adequate volume of NaOH slowly through the column and rinsing with distilled water to remove excess sodium ions



 The resin can have a capacity of 1 g of streptomycin g-l resin. Commercially, it is not economic to regenerate the resin completely, therefore the capacity will be reduced. In practice, the filtered broth is taken through two columns in series while a third is being eluted and regenerated. When the first column is saturated, it is isolated for elution and regeneration while the third column is brought into operation

Ion-exchange chromatography may be combined with HPLC in, for example, the purification of somatotropin using DEAE cellulose columns and β-urogastrone in multi-gram quantities using a cation exchange column

**GEL PERMEATION**

This technique is also known as gel exclusion and gel filtration. Gel permeation separates molecules on the basis of their size. The smaller molecules diffuse into the gel more rapidly than the larger ones, and penetrate the pores of the gel to a greater degree. This means that once elution is started, the larger molecules which are still in the voids in the gel will be eluted first. A wide range of gels are available, including cross-linked dextrans (Sephadex and Sephacryl) and cross-linked agarose (Sepharose) with various pore sizes depending on the fractionation range required.

One early industrial application, although on a relatively small scale, was the purification of vaccines (Latham *et al*., 1967). Tetanus and diphtheria broths for batches of up to 100,000 human doses are passed through a 13 dm3 column of G 100 followed by a 13 dm3 column of G 200. This technique yields a fairly pure fraction which is then concentrated ten-fold by pressure dialysis to remove the eluant buffer (Na2 HP04)·

**AFFINITY CHROMATOGRAPHY**

Affinity chromatography is a separation technique with many applications since it is possible to use it for separation and purification of most biological molecules on the basis of their function or chemical structure.

This technique depends on the highly specific interactions between pairs of biological materials such as enzyme-substrate, enzyme-inhibitor, antigen-antibody, etc. The molecule to be purified is specifically adsorbed from, for example, a cell lysate applied to the affinity column by a binding substance (ligand) which is immobilized on an insoluble support (matrix).

Eluent is then passed through the column to release the highly purified and concentrated molecule. The ligand is attached to the matrix by physical absorption or chemically by a covalent bond. The pore size and ligand location must be carefully matched to the size of the product for effective separation.

Coupling procedures have been developed using cyanogen bromide, bisoxiranes, disaziridines and periodates, for matrixes of gels and beads. Four polymers which are often used for matrix materials are agarose, cellulose, dextrose and polyacrylamide. Agarose activated with cyanogen bromide is one of the most commonly used supports for the coupling of amino ligands. Silica based solid phases have been shown to be an effective alternative to gel supports in affinity chromatography.

Purification may be several thousand-fold with good recovery of active material. The method can however be quite costly and time consuming, and alternative affinity methods such as affinity cross-flow filtration, affinity precipitation and affinity partitioning may offer some advantages.

Affinity chromatography was used initially in protein isolation and purification, particularly enzymes. Since then many other large-scale applications have been developed for enzyme inhibitors, antibodies, interferon and recombinant proteins and on a smaller scale for nucleic acids, cell organelles and whole cells

**REVERSE PHASE CHROMATOGRAPHY (RPC)**

This chromatographic method utilizes a solid phase (e.g. silica) which is modified so as to replace hydrophilic groups with hydrophobic alkyl chains. This allows the separation of proteins according to their hydrophobicity.

More-hydrophobic proteins bind most strongly to the stationary phase and are therefore eluted later than less-hydrophobic proteins. The alkyl groupings are normally eight or eighteen carbons in length (C8 and C18) RPC can also be combined with affinity techniques in the separation of, for example, proteins and peptides.

**HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)**

HPLC is a high resolution column chromatographic technique. This allows packing in columns with minimum spaces between the beads, thus minimizing peak broadening of eluted species. It was originally known as high pressure liquid chromatography because of the high pressures required to drive solvents through silica based packed beds.

HPLC is distinguished from liquid chromatography by the use of improved media (in terms of their selectivity and physical properties) for the solid (stationary) phase through which the mobile (fluid) phase passes.

The stationary phase must have high surface area/unit volume, even size and shape and be resistant to mechanical and chemical damage. However, it is factors such as these which lead to high pressure requirements and cost. This may be acceptable for analytical work, but not for preparative separations. Thus, in preparative HPLC some resolution is often sacrificed (by the use of larger stationary-phase particles) to reduce operating and capital costs. For very high value products large-scale HPLC columns containing analytical media have been used.

**CONTINUOUS CHROMATOGRAPHY**

Although the concept of continuous enzyme isolation is well established (Dunnill and Lilly, 1972), the stage of least development is continuous chromatography. Fox et al. (1969) developed a continuous-fed column for this purpose (Fig. 10.30).

It consisted of two concentric cylindrical sections clamped to a base plate. The space (1 cm wide) between the two sections was packed with the appropriate resin or gel giving a total column capacity of 2.58 dm3.

A series of orifices in the circumference of the base plate below the column space led to collecting vessels.

The column assembly was rotated in a slow-moving turntable (0.4-2.0 rpm). The mixture for separation was fed to the apparatus by an applicator rotating at the same speed as the column, thus allowing application at a fixed point, while the eluent was fed evenly to the whole circumference of the column. The components of a mixture separated as a series of helical pathways, which varied with the retention properties of the constituent components. This method gave a satisfactory separation and recovery but the consumption of eluent and the unreliable throughput rate were not considered to be satisfactory for a large-scale method.

However, the development of such continuous separation equipment suitable for large scale extraction would considerably simplify the use of chromatographic separation.

References

1. Stanbury, The recovery and purification of fermentation product. Principle of fermentation technology. Second edition. Butterworth-Heinemann