

The Purification of Microbial Biocatalysts by Filtration and Ammonium Sulphate Precipitation



image courtesy of Cuno Limited

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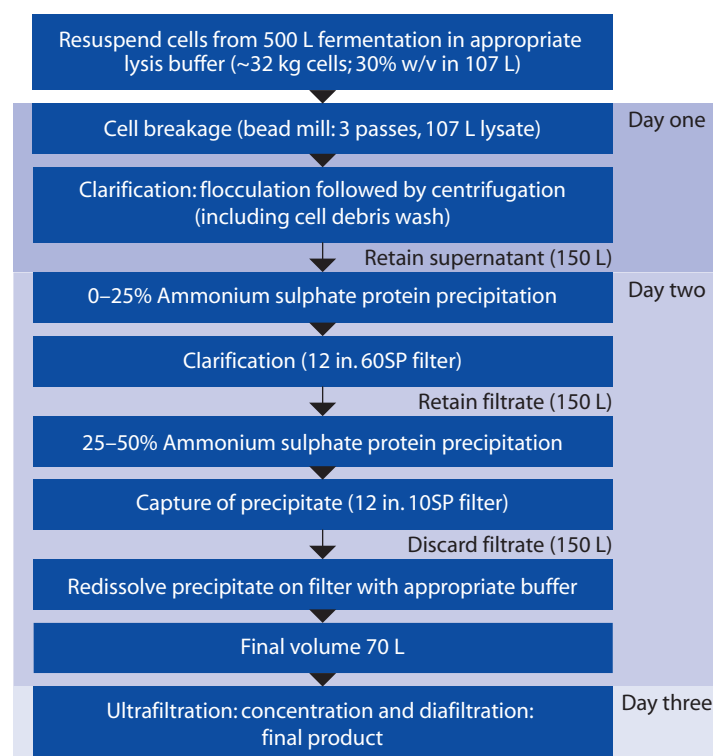
This paper describes the purification of an enzyme from a recombinant strain of *Escherichia coli* using lenticular filters, and focusses on the development of ammonium sulphate protein precipitation stages. Following evaluation studies, the laboratory filters were subsequently scaled to pilot and then full-scale. This research has established a generic route for the production of large-scale quantities of an isolated enzyme to be used for industrial applications, such as the resolution of racemic mixtures of amino acids using acylase enzymes.

Enzymes are used for the synthesis of chiral compounds. One important consideration is the cost of producing the enzymes, which can be divided into the research and development (R&D) costs and subsequent production batch costs. The semi-purified enzyme is deemed a raw material for the subsequent biocatalysis reaction, so the cost of producing the enzyme has to be low. To achieve the overall economic target, investment is made into the screening of improved biocatalysts. Once this has been achieved, the selected enzyme is subsequently cloned for over-expression in an organism such as *Escherichia coli*. By implementing this method, up to 40% (w/w) of the total cell protein can be expressed and released as the target enzyme. A robust, quick

and efficient process for the isolation of the target enzyme also reduces the overall cost.

The preparation of large quantities of relatively pure enzyme is often difficult for small- to medium-size biopharmaceutical/biotechnology companies to achieve. Availability of equipment, allocated time to develop a process and the cost to produce the catalyst with respect to the overall costs of the final product are typical constraints. Many organizations purchase harvested cells or crude cell lysates that may be subsequently purified to improve the quality and increase the specific activity of the preparation (enzyme activity per mg protein). Purification reduces common problems associated with using crude lysates in biotransformation reactions, where

Figure 1 A flow schematic of a typical biocatalyst downstream process.



emulsions often form between protein solutions and organic solvents. Emulsions can be extremely difficult to remove, particularly when scaling to kilogram reactant quantities.

A high quality purified protein has more potential applications. One example is the immobilization of the target enzyme onto solid supports, further aiding the biotransformation by allowing the biocatalyst to be recycled.

This paper describes the development of a generic method for purifying a number of industrially important enzymes (biocatalysts) used in large-scale biotransformations. The significant purification steps in the downstream process use ammonium sulphate protein precipitation and two types of lenticular filters. The first filter is used to clarify the material; the second to capture the precipitated protein, which is then recovered from the filter by flushing with fresh buffer, thereby redissolving the precipitate.

Development of the ammonium sulphate precipitation steps

Ammonium sulphate precipitation is well understood, but the types and

grades of filters that are used in conjunction with it need to be optimized to provide the necessary quality, yield, acceptable processing time and ease of

operation. This leads to an economic and viable process. In this study, filtration was employed at two steps, first to clarify and secondly to recover the precipitated protein. The two filtration steps have distinctly different requirements, but a similar method was used to optimize the filters. Many different types of filter medium are available, but one that utilizes all of the filtration mechanisms gives the optimum process with respect to quality and cost.

A variety of different porosity filter media (Zeta Plus 05SP, 10SP, 30SP, 50SP and 60SP; Cuno Limited) in a disposable filter (Biocap 30; Cuno) were tested for the clarification and precipitation stages. These filters are composed of a cellulose-based medium that contains filter aid and is bound together by a positively charged resin. This provides particle retention by size exclusion (particles retained on or near the surface) and entrapment (particles retained

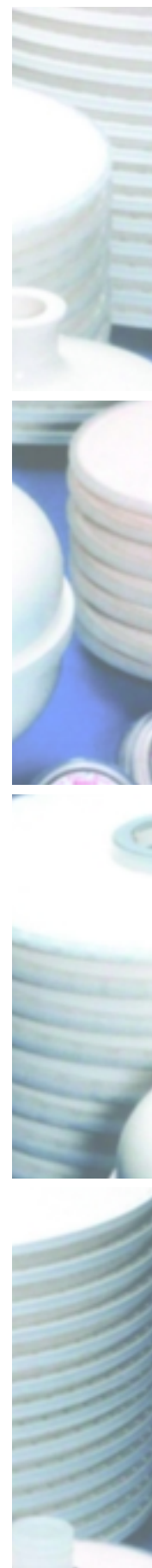
within the medium) by impaction, diffusion and electrokinetic capture mechanisms. The electrokinetic charge allows smaller particles to be retained, and the filters have an open structure providing good flow rates. Protein binding is usually not significant in the early clarification stages.

Clarification. The clarification stage was investigated first. The more open 10, 30 and 50SP grades resulted in the product having a haze. The optimum grade for clarity was found to be the more retentive 60SP grade medium, which was then used for throughput studies. Throughput studies were used in preference to V_{\max} pore-plugging optimization studies because the structure of the medium does not obey the pore-plugging mechanisms upon which the V_{\max} model is based. The optimum throughput was found to be 90 cm³ per 47 mm diameter disc, equivalent to 6.7 cm³cm⁻². In a scaled-up trial, an 8 in. cartridge with a surface area of 3000 cm² provided 14 L of throughput (4.7 cm³cm⁻²). These data are more reliable for final scale-up than data from the 47 mm diameter discs. For production, this was later scaled to two 12 in. filters having a total surface area of 36000 cm² and the ability to process 168 L of material.

Precipitation. The precipitate recovery step showed that using the 05SP grade resulted in protein passage through the filter. This filter medium showed good retention of the biocatalyst with 95% recovery and total throughput of the recovery stage was 97 cm³ per 47 mm diameter disc (7.2 cm³cm⁻²). This was scaled-up to an 8 in. cartridge to provide accurate data for production. All studies used typical batches from production to ensure linear scaling.

Process-scale production of the biocatalyst

The downstream process takes approximately 2–3 days from cell lysis through to the preparation of final product. During this process, the temperature is monitored and maintained between 4 and 12 °C, where possible, to preserve activity. Figure 1 and Table I show the recovery of the enzyme during downstream processing.



The harvested recombinant *E. coli* cells from a 500 L (32 kg) fermentation were resuspended in a buffer to a cell density of 30% (w/v) — the optimal cell density for efficient cell lysis by means of a bead mill (KDL Eco 5 Bead Mill, Glen Creston, Stanmore, UK). The cell lysate generated from bead milling was then treated with a commercially available flocculent (overnight contact) to help at a later clarification stage. This cell lysate was processed through a Westfalia separator CSA8-47-476 continuous centrifuge (Milton Keynes, UK). The cellular debris from the first pass was resuspended in buffer, mixed for 1 h and re-centrifuged to obtain the maximum amount of enzyme product in the clarified lysate.

The clarified lysate (supernatant) obtained from centrifugation (150 L) was then treated with ammonium sulphate to 25% (w/v) saturation. The protein precipitate was encouraged to form with gentle mixing for 1–2 h. The resulting precipitate was

then filtered using two 12 in. 60SP cartridges contained within a stainless steel filter housing.

The resulting filtrate, containing the target protein, was collected and subjected to a second ammonium sulphate protein precipitation step (50% [w/v] overall saturation). In this step, the target protein was precipitated upon gentle mixing for 1–2 h. The solution was passed through the 10SP filters (2 × 12 in. cartridges) using a peristaltic pump. The filter retained the protein precipitate and the filtrate (approximately 150 L) was discarded. Once the solution had passed through, the filter was pumped free of protein solution using compressed air. The filtrate was tested for enzyme activity and only 11% of the target activity was found. A solution of fresh buffer was then recirculated through the system for 1–1.5 h to fully redissolve the ammonium sulphate-precipitated protein ‘cake.’ A step recovery of 71% was achieved. The final solution (approximately 70 L) was collected at the outlet of the filter

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housing and then concentrated and/or diafiltered to remove the ammonium sulphate. A final overall recovery of 57% was observed. This final product would be suitable for use in biotransformation experiments.

The first clarification step showed almost 80% recovery (see Table I). The loss may result from enzyme being bound to the cell debris that is subsequently retained by the filter. The second filtration precipitation step showed enzyme activity in the discarded filtrate of only 11% and the recovery was 56%. Activity may also have been lost as a result of activators associated with the enzyme being removed, or because of volume losses in processing. On removal of the ammonium sulphate

Table I Typical downstream processes for biocatalyst purification.

Sample description	Volume (L)	Activity (units/cm ³)	Protein (mg/cm ³)	Total activity (× 1000)	Specific activity (U/mg)	Process recovery (%)	Step recovery (%)	Purification
Bead mill pass 1 cell-free extract	75	1664	32.65	124820	51.0	76.8	–	1.0
Bead mill pass 2 cell-free extract	75	2076	43.81	155731	47.4	95.8	124.8	0.9
Bead mill pass 3 cell-free extract	75	2168	46.12	162581	47.0	100.0	104.4	0.9
Post-flocculent supernatant	75	2113	44.10	158450	47.9	97.5	97.5	0.9
Centrifuge supernatant	150	1136	21.08	170372	53.9	104.8	107.5	1.1
0–25% (NH ₄) ₂ SO ₄ filtrate (60SP filter)	150	877	15.78	131598	55.6	80.9	77.2	1.1
25–50% (NH ₄) ₂ SO ₄ redissolved pellet (10SP filter)	70	1362	12.57	90945	108.4	55.9	69.1	2.1
Waste filtrate 25–50% (NH ₄) ₂ SO ₄ (10SP filter)	150	122	6.70	18324	18.2	11.3	20.1	0.4

Definitions

- Cell-free extract refers to the supernatant obtained after having removed the cell debris from the lysis mixture. In all cases, activity is calculated from this ‘soluble’ fraction. Enzyme may be associated with cell debris and cell walls, for example, but only enzyme in the soluble fraction can be processed further so all assays refer to the soluble activity.
- Process/recovery (%) refers to amount of activity at each step in comparison with, in this case, the amount of activity obtained from three passes through the bead mill (referred to as 100%)
- Step recovery (%) refers to the recovery of enzyme activity compared with the previous step, for example, 69.1% of enzyme activity was recovered from 10SP filters.
- Purification refers to the increase in specific activity, for example, increase in activity compared with amount of protein.

(by diafiltration) overall recovery of activity of up to 75% was observed (data not shown) — that is, ammonium sulphate was inhibiting the enzyme activity.

The final filters used in the recovery of the protein can be reused to process further batches, or to repeatedly process partial batches, to reduce process costs. The filter can be used until a differential pressure

of 2 bar is reached; this pressure drops dramatically once the second buffer is applied to the filters to dissolve the precipitate, and so repeated use is a possibility.

Acknowledgement

This work was conducted by CUNO's Scientific Application Support Services (SASS). ■

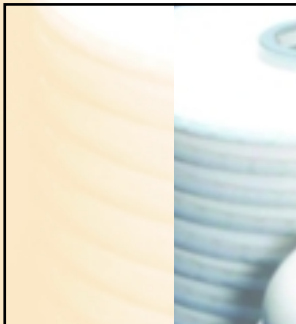
Article reprinted from the
©June 2001 issue of:

**Pharmaceutical
Technology**
EUROPE

Reprint Publication # 0472



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