Comparative Evaluation of Different Methods of Extraction and Purification Used in Technical Enzyme Production from Microorganisms

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Abstract

A study was conducted to compare the efficiency of two methods of microbial cell separation and enzyme purification using penicillin-G-acylase (PGA) from Escherichia coli. The efficiency of two methods of cell separation; centrifugation and cross flow filtration (CFF) were compared. The CFF method was found to have both higher separation efficiency and enzyme yield than centrifugation method. Centrifugation method gave a separation efficiency of 98.5% with enzyme yield of 94% whereas CFF method resulted in 100% separation efficiency and enzyme yield of 98.8%. The ***Escherichia coli*** *cells were disrupted by high pressure homogenization (HPH) and the disrupted cells were purified using two different techniques. Technique I was a combination of cross-flow-diafiltration (CFD), ultrafiltration (UF) and heat/pH-shift treatments. This technique resulted in 47% enzyme yield with a purification factor of 12. Technique II which involved two extraction steps by aqueous two - phase system (APS) coupled with UF resulted in 62% enzyme yield with a purification factor of 4. Technique I was therefore much better than technique II in purifying the enzyme. For higher enzyme yield, technique II would seem to be a better one than technique I.*

Keywords: Enzyme yield, microbial enzymes, purification factor, recovery rate, separation efficiency, and technical enzyme

Introduction

Enzymes are widely used in the food, chemical and pharmaceutical industries. At present it is estimated that out of more than 2,500 known enzymes, about 50 are manufactured at an industrial scale, mainly from micro-organisms. More applications of enzymes in food, chemical and pharmaceutical industries and possibly others is expected to increase with the advancement in biotechnology. One of the pre-requisites for the economic production of enzymes is to have an efficient recovery and purification process. According to Kroner (1994), these processes involve a series of consecutive separation steps that can be categorized as the primary separation, enrichment and purification. The primary separation mainly involves the removal of cells and cell debris from homogenate whereas the enrichment step deals with the pre-purification and concentration processes. The purification step is directed to achieve high purity. The number of steps involved in the whole process determines

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the enzyme yield and the degree of purity, and therefore the cost of enzyme production. For economic reasons, it is advisable to keep the number of steps as low as possible, but this has to take into consideration the required degree of purity and activity for the enzyme in question. Fortunately, for the technical grade enzyme production, a very high degree of purification is often not needed, and as such the process development is focused mainly on an efficient enrichment at high yields but taking into account the removal of interference to the enzyme activity (Hustedt *et al.*, 1985).

Various methods are available for the enrichment of enzymes. However, due to their differences in principles and operational techniques, they vary in the enzyme recovery and purification performances. The selection of a combination of suitable methods is therefore very important in the production of enzymes. Most of the methods used in enzyme extraction and purification involve conventional techniques such as centrifugation, cell disruption, precipitation with ammonium sulphate, ion exchange or other types of adsorption methods (Schütte and Kula, 1990; Shewale and Sivaraman, 1989). Alternative methods such as Cross-Flow Filtration (Kroner *et al*, 1984; Kroner, 1994), extraction with aqueous phase systems (Hustedt *et al*, 1985) have also been used.

In this study, two conventional methods of separation and two alternative techniques (techniques I and II) for the extraction and purification of enzymes were evaluated using Penicillin-G-acylase as a technical enzyme. Penicillin-G-acylase (PGA) is widely used for the commercial production of 6-aminopenicillanic acid (6-APA), the basic material for the manufacture of semi-synthetic penicillin (Desphpande *et al*, 1994).

Materials and Methods

Escherichia coli 5K (pHM 12) Cultivation

Escherichia coli 5K (pHM 12), DSM 4760 (DSM, 1993) was used for penicillin-G-acylase production. The pre-culture was prepared by using a 12 hour starter culture of medium NMB which was transferred to a 15-L fermenter containing 10 L of medium NM6. The fermentation process was run at 27 °C, pH 6.8 with stirrer speed of 400 rpm and an aeration rate of 5 L/min for 12 hours. Pre-culture was then used to inoculate three-150 L fermenters containing 100 L of medium NM7 each. The *E. coli* cells were cultivated for 12 hours. The composition of media NMB, NM6, and NM7 is shown in Table 1.

Table 1: Composition of different media used for cultivation of *Escherichia coli* 5K (pHM 12)

	Medium		
Component	NMB	NM6	NM7
Yeast extract	13.0g/l	15.0 g/l	30.0 g/l
Malic acid	-	-	2.5 g/l
Glucose	-	6.0 g/l	-
NH_4Cl	2.4 g/l	-	-
KH_2PO_4	6.8 g/l	-	-
$NaHPO_4.2H_2O$	8.9 g/l	-	-
Tetracycline HCl	4.0 mg/l	4.0 mg/l	4.0 mg/l

Cell Harvesting

Escherichia coli cells were harvested by centrifugation using Westfalia separator CSA-8, Type-06-476 and by cross-flow filtration using Sartocon II, plate and frame system. The degree of clarification was pre-determined by varying the feed rate and by measuring the Optical Density ($A_{546}nm$) of the overflow. The highest separation efficiency (98.5%) was achieved at the feed rate of 100-200 l/hour The cell harvesting was then performed at the feed rate of 200 l/hour with the running time of 72 minutes. The biomass (sludge) from each method were collected and analyzed for separation efficiency and enzyme yield. The biomass from centrifugation and cross-flow filtration methods was then pooled.

Cell Disruption

Of the two methods of microbial cell disruption as recommended by Kroner (1994), HPH was found to be superior to sonication and was therefore adopted for use in this study. The cells were disrupted by high pressure homogenization using APV-Gaulin, Type Lab 60/60-10 TBS homogenizer. The cell homogenate was then divided into 2 portions. One portion was used for evaluating purification efficiency of the combination of cross-flow diafiltration, ultrafiltration and heat-and pH-shift treatment methods (Technique I). The other portion was used for the evaluating purification efficiency by a two-phase aqueous system (APS) coupled with ultrafiltration (Technique II). Further purification of samples from APS was carried out by hydrophobic interaction chromatography (HIC) and characterized by SDS-PAGE gel-electrophoresis (Figure 1).

Enzyme Isolation and Purification

Technique I

Cross-Flow Diafiltration and Ultrafiltration

In order to set up the optimum operational conditions for the large-scale cross-flow diafiltration, a small scale experiment was performed by varying the filtrate rate. The optimum filtrate rate of about 1.4 l/hour corresponding to pressure of about 0.8 bar with 66% enzyme transmission was selected. Selecting higher pressure resulted in prolonging the experimental running time.

Heat- and pH- Shift Treatments

The retentate from the UF was subjected to heat- and pH-shift treatments after the suitable conditions were established by using a small amount of the same retentate. The results of the small scale experiment revealed that combination of heat at 55^{o}C and pH 5.0 gave the highest specific enzyme activity. This condition was consequently used to set up the large scale purification.

Technique II

Extraction by Aqueous Two-Phase System (APS)

The extraction and enrichment of enzyme is commonly a two-step procedure by APS (Buntemeyer et al., 1989). The first step to remove cell debris (APS I) and the second step (APS II) is to re-extract the enzyme from the first top phase. A small scale experiment was conducted to determine the suitable condition in the first step (APS I) by varying the phosphate concentration (6.6%, 7% and 7.5%). The conditions with the phosphate concentration of 7% and 7.5% gave similar enzyme yield in the top phase. However, 7% phosphate concentration was

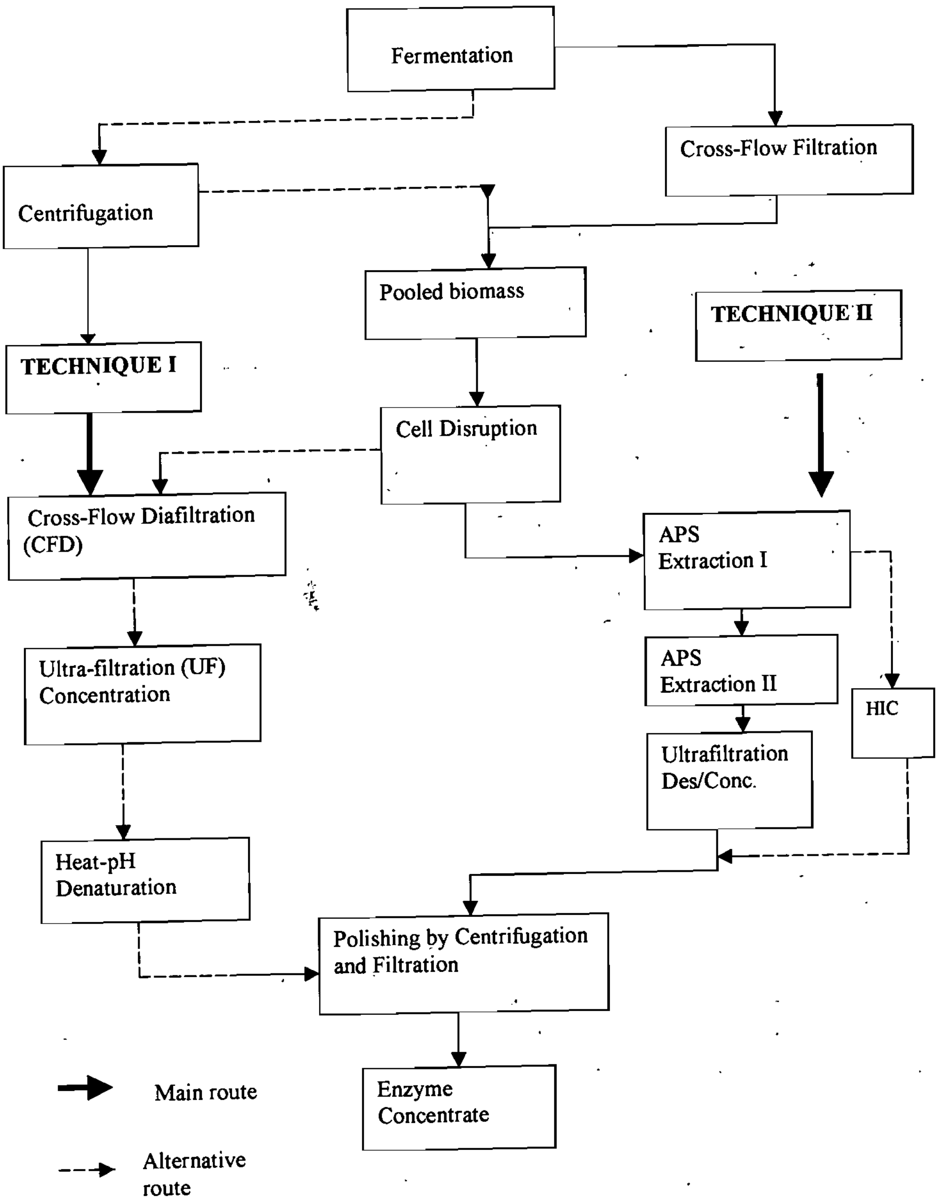

Figure 1: Flow scheme of recovery of Penicillin G Acylase (PGA) from *Escherichia coli* 5K, DSM4760 (pHM 12)

chosen for the first extraction in large scale experiment due to the fact that it gave lower enzyme yield in the bottom phase. The small amount of the top phase of the large scale was then used to determine the suitable pH for the second step (APS II) by varying NaOH concentration (0.24%, 0.28% and 0.32%). The maximum enzyme yield (79%) in the bottom phase was obtained with 0.24% NaOH and this was then used for the second extraction (APS II) in the large scale.

PGA Purification by Hydrophobic Interaction Chromatography (HIC)

Further PGA purification was performed by using hydrophobic interaction chromatography. Small amount of the sample from APS was directly applied on HIC column and chromatogram was obtained.

Analytical Procedure

The activity of PGA obtained at different stages of the isolation procedure was determined by the NIPAB method (Kutzbach and Rauenbusch, 1974). Protein measurement was carried out by the Bradford method (Bradford, 1976).

Results and Discussion

Fermentation

The results of *E. coli* 5K (PMH12), DSM 4760 cultivation in 3 different 150 litre bioreactors were as shown in Table 2. The fermentation broth from bioreactor F150.1 and F150.2 gave more or less the same optical densities. However, the optical density of the bioreactor F150.3 was relatively lower than that of other two bioreactors probably because of some defects in the bioreactor. Similarly, the PGA activity achieved corresponded to the amount of biomass at the end of cultivation. The enzyme activity in the supernatant of the fermentation broth during the 12 hour cultivation was less than 1 U/ml. This indicates good cell stability during cultivation. Similar observations were also reported by Kroner *et al.*, (1984) although the actual figures they gave were smaller.

Cell Harvesting

Centrifugation Method

The process of centrifugation resulted in enzyme yield of about 94% with about 1% loss in enzyme yield in the overflow (Table 3). Enzyme yield of about 15% was also observed in the supernatant of the sludge (cell biomass). This could be due to the cell damage during

Table 2: Cultivation of *Escherichia coli* 5K DSM 4760 (pHM 12) in three different bioreactors

Bioreactor	OD (546nm)	PGA2 (U/ml)	Protein (mg/ml)	Sp. Activity (U/mg)	Wet weight (g/kg)
F150.1	9.465	0.614	3.836	0.160	23.32
F150.2	9.066	0.795	3.793	0.210	23.95
F150.3	3.689	0.244	1.170	0.209	13.39
Pool[1]	9.043	0.730	2.570	0.284	26.30

[1]pool is the mixture of the fermentation broth from 3 bioreactors Cells were disrupted by sonication for 3 minutes
OD = optical density
Sp. Activity = specific activity
PGA2 = PGA activity

Table 3: Comparison of effeciencies of different methods used in microbial cell harvesting during enzyme production

Method	Volume (L)	Average flow rate (L/h)	Equ.Flux/area[1] (L/h eq.A)	STY2 (kg/Lh)	PGA (kg/Lh)	Cell yield (%)	Separation efficiency[3] (%)	Degree of concentration
Centrifugation (CSA-8)	235.00	200.00	29.70	11.36	94.00	96.70	98.60	23.10
Cross Flow Filtration (CFF)	64.00	51.20	55.70	12.81	98.80	100.00	100.00	18.80

[1]Taking into account that 1000cm^3 of separator corresponds to 1 m^2 of membrane area (Factor CSA8/CFF = 7.3)
[2] STY = Space - Time - Yield (in terms of kg mass or unit processed) = kg wt cell mass /
[3]Ratio of overflow of filtrate turbidity/feed turbidity
Kg/Lh = Amount harvested in kg per litre per hour

centrifugation. A concentration of about 23% of the cell biomass was achieved by this method.

Cross Flow Filtration method (CFF)

Cell harvesting by CFF method resulted in nearly 100 % enzyme yield in the sludge and about 1% in the filtrate. This indicates that less cells were damaged during CFF. However, in terms of concentrating cell biomass, this method could only concentrate the sample to nearly 19% (Table 3).

Comparing the two methods of cell harvesting (Centrifugation and CFF), both of them seem to give more or less the same Space Time Yield (STY). However, the separation efficiency and enzyme yield by CFF were higher than that of Centrifugation (Table 3).

PGA Purification Technique I

Cross-Flow Diafiltration and Ultrafiltration

For the large scale experiment, the calculated optimal filtrate rate to be used was 18 l/hour. During filtration (5 times wash volume), the filtrate rate was however not steady, it fluctuated from 15.8 to 18.2 l/hour. The maximum enzyme yield achieved by this method was about 90.5%. The filtrate from diafiltration was then concentrated by ultrafiltration (UF). The final enzyme yield obtained was about 85%, a 5.5% decrease in the enzyme yield but with an increase in the purification factor from 1.2 to 1.8 (Table 4).

Heat- and pH- Shift Treatments

The overall PGA purification by cross-flow diafiltration followed by heat- and pH-shift treatment (technique I) resulted in about 51% enzyme yield with the final purification factor of about 12 (Table 4). This result shows that only 50% enzyme recovery is possible by this technique. Most of the enzyme loss was encountered in the step of heat- pH-shift treatment. According to Büntemeyer, *et al.*, (1989), this could probably be due to the enzyme denaturation in the lag period in attaining optimum temperature and pH.

PGA Purification Technique II

Extraction by Aqueous Two-Phase System (APS)

Table 4 shows the results of the two-step APS extraction. About 97% and 71% enzyme yield were obtained in the APS I and APS II respectively. About 4% of the enzyme yield was also observed in the inter-phase indicating that the phase separation was not complete. During the APS extractions, the purification factor improved from 3.1 in the APS I to 3.7 in APS II (Table 4). The bottom phase of the

Table 4: Comparison of effeciencies of different steps in industrial technical enzyme production from *E. Coli* 5K, DSM 4760

Technique	Purification Step	Volume (L)	PGA (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	PGA yield (%)	Protein yield (%)	Purification factor
I	Diafiltration	51.30	1.18	2.87	0.41	90.50	55.30	1.20
	Ultrafiltration	2.80	22.21	36.27	0.61	85.40	46.90	1.80
	Heat and pH shift	2.40	15.67	3.99	3.93	51.30	'4.40	11.60
II	APS I_{top}	11.99	11.31	10.92	1.40	96.90	31.50	3.10
	$APS_{interphase}$	1.30	0.47	0.77	ND	3.60	1.20	ND
	APS II_{bottom}	11.81	8.37	6.69	1.25	70.60	19.00	3.70
	Ultrafiltration	1.00	88.05	62.78	1.40	62.90	15.10	4.10

ND = Not done

APS II was further subjected to ultrafiltration to remove the salt as well as to concentrate the sample. The overall PGA purification by APS followed by UF resulted in 63% enzyme yield with the final purification factor of 4.1 (Table 4). Most of the enzyme loss was encountered during APS separation. This is possibly due to denaturation of the enzyme caused by sudden change in ionic strength and pH during mixing (Hustedt *et al.*, (1985).

PGA Purification by Hydrophobic Interaction Chromatography (HIC)

Further PGA purification from APS II and UF was performed by using hydrophobic interaction chromatography (HIC) as described by Kutzbach and Rauenbusch (1974). According to the same authors, the highest enzyme activity was observed from fraction no. 13 corresponding to the peak with the retention time of 38.15 minutes. When the fraction with the highest enzyme activity (no.13) was further subjected to SDS-PAGE along with samples from the APS purification steps prior to HIC application, the results showed that the purity of sample increased tremendously by using HIC.

Conclusions

The overall performance of technique I, involving cross-flow diafiltration, ultrafiltration and heat-and pH-shift treatment, resulted in 51% of enzyme yield with purification factor of 12. Technique II, which involved two-phase aqueous system (APS) coupled with ultrafiltration resulted in 63% enzyme yield and purification factor of 4. From the point of view of enzyme yield, technique II gave a higher yield than technique I although its purity was comparatively low. Selection of the suitable technique will depend on the required specifications of the technical enzyme: If high enzyme yield is preferred to high purity, technique II would seem to be a better one than technique I and vice versa. Higher enzyme yield and purity can be achieved by technique II followed a further step of HIC.

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