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Simulation of Large-Scale Production of a Soluble Recombinant Protein Expressed in *Escherichia coli* Using an Intein-Mediated Purification System

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Abstract

Inteins are self-cleavable proteins that under reducing conditions can be cleaved from a recombinant target protein. Industrially, an intein-based system could potentially reduce production costs of recombinant proteins by facilitating a highly selective affinity purification using an inexpensive substrate such as chitin. In this study, SuperPro® Designer was used to simulate the large-scale recovery of a soluble recombinant protein expressed in *Escherichia coli* using an intein-mediated purification process based on the commercially available IMPACT® system. The intein process was also compared with a conventional process simulated by SuperPro. The intein purification process initially simulated was significantly more expensive than the conventional process, primarily owing to the properties of the chitin resin and high reducing-agent (dithiothreitol [DTT]) raw material cost. The intein process was sensitive to the chitin resin binding capacity, cleavage efficiency of the intein fusion protein, the size of the target protein relative to the intein tag, and DTT costs. An optimized intein purification process considerably reduced costs by simulating an improved chitin resin and alternative reducing agents. Thus, to realize the full potential of intein purification processes, research is needed to improve the properties of chitin resin and to find alternative, inexpensive raw materials.

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Index Entries: Chitin; process simulation; recombinant protein production; intein; economic analysis.

Introduction

The translation of a successful laboratory-scale process into an economically feasible industrial process often requires additional research. Bioprocess simulators aid in uncovering such aspects of the process that require further optimization and can be used to conduct economic assessment at an industrial scale by incorporating laboratory scale data *(1–3)*. Intein-Mediated Purification with an Affinity Chitin-binding Tag (IMPACT® system; New England Biolabs, Beverly, MA) is currently used to facilitate the effective recovery of a recombinant protein in a single step at the laboratory scale. This system has been successfully used at the bench scale to express and easily purify stable proteins in which activity was maintained *(4–7)*.

The IMPACT system expresses the recombinant target protein in fusion with a self-cleaving protein (intein). The intein, in turn, is fused to or has inserted into it a chitin-binding domain (CBD) that serves as an affinity tag. This CBD permits isolation of the fusion protein on a chitin column. The target protein is then recovered by inducing its cleavage from the intein tag under reducing conditions. The IMPACT system is currently the only commercially available intein purification system; however, several other intein-based systems have been developed using different inteins and affinity tags *(5,8–13)*.

The objective of the present study was to determine the economics of a large-scale process for the production of a generic soluble recombinant protein (GRP) from *Escherichia coli* using the intein purification system. A generic soluble recombinant protein was selected as the target protein to be purified, because fusion of a protein with an intein has been known to promote its expression in a soluble form *(14,15)*. The intein purification process simulated in our study used a modified *Mycobacterium xenopi gyrase* A (*Mxe GyrA*) intein, fused to a CBD (New England Biolabs). As a benchmark, a conventional process for the purification of the same soluble recombinant protein was designed. A molecular mass of 25 kDa was selected for the GRP based on the size of a typical therapeutic recombinant protein produced in *E. coli* and approved by the Food and Drug Administration (FDA). The simulations were not protein specific. For these simulations, only the cellular production capacity based on the size of the target protein relative to the intein protein affected the cost estimates. This effect was also investigated in the study. The use of a benchmark conventional process allowed direct comparison between the two processes using identical assumptions. Specifically, the raw material costs were identical, as were the equations used to calculate capital cost, operating cost, depreciation, and other economic parameters. The operating specifications of the process equipment were also identical. Comparison with conventional pro-

cesses currently in production was not undertaken because these processes would have different cellular productivity and cost basis assumptions.

Many commercial recombinant expression systems result in the recombinant protein being expressed as inclusion bodies that must be purified using chaotropic agents and refolding steps. The formation of inclusion bodies cannot be predicted for a particular protein *(16)*. Inclusion bodies also do not necessarily protect the recombinant protein from proteases *(16,17)*. The additional steps associated with the purification of a protein from inclusion bodies increase protein loss and purification costs. Thus, for our study, the intein and conventional processes were simulated to produce a soluble recombinant protein. Other studies have shown that for equal expression levels, a conventional inclusion body process is more expensive than a conventional soluble protein process *(18)*.

SuperPro® Designer simulation software was used to compare the conventional and intein-mediated processes. Each process was designed to meet the purification objectives for a recombinant biopharmaceutical *(19,20)*. The conventional purification process utilized classic purification steps: ion exchange chromatography, hydrophobic interaction chromatography (HIC), and gel filtration chromatography. The intein-mediated purification process included intein/chitin expanded-bed chromatography, ion-exchange chromatography, and gel filtration chromatography. These process simulations allowed detailed analysis of the intein process economics, and the conventional process provided benchmark economics. A sensitivity analysis of the intein process was conducted, and where applicable, a parallel analysis of the conventional process. The sensitivity analysis indicated where process improvements would have the greatest economic impact.

Process Design

The purification processes were developed based on data collected from work conducted at the bench scale *(21)*. For the simulated intein process, the 25-kDa recombinant protein was fused to the N-terminus of a modified *Mxe GyrA* intein (23 kDa), and a CBD (5 kDa). The modified intein had an amino acid substitution, which prevented in vivo cleavage of the fusion protein, but allowed the intein to catalyze thiol-inducible cleavage at its N-terminal in vitro. The simulated intein purification steps closely approximated the intein system as described in established protocols *(5,8)*. It was assumed that the intein purification of the recombinant protein had not been optimized. Likewise, the recombinant protein produced in the conventional process used general rules-of-thumb guidelines and was not rigorously optimized.

The intein and conventional processes were designed based on experience with the purification of numerous recombinant proteins, as well as literature and patent data *(18,22–27)*. Both processes were designed to produce 100 kg of GRP/yr, to allow economic comparison between the two

purification methods. Since it was assumed that equal quantities of recombinant protein were expressed by the cells (5% of the total dry cell weight), 50 mg of the GRP was produced for every gram of dry cell weight in the conventional process, whereas 50 mg of the intein fusion protein was produced per gram of the dry cell weight. This translated into only 24 mg of GRP produced for every gram of dry cell weight for the intein process, because only 47% of the intein fusion protein was the target (GRP). Consequently, the amount of biomass required for the production of a particular quantity of the target protein for the intein process was significantly higher than for the conventional process. The simulations were run in design mode such that the sizing of the equipment was completed by SuperPro. The design parameters for the equipment in both processes, such as percentage of rejection in the filtration units and capture efficiency in the ion-exchange and gel filtration units, were identical. For the ion-exchange, gel filtration, and hydrophobic chromatography resins, conservative separation efficiencies, reusability, and cost estimates were used. The binding capacity and replacement frequency of the chitin resin were selected based on values obtained from laboratory studies (5 mg/mL and five cycles, respectively). These values were significantly lower than for most commercially available affinity resins. Cleavage efficiency of the intein from the target protein was 60%, which was well within experimentally observed values. Process scheduling and labor requirement calculations were performed using tools built into SuperPro Designer. The material flow of the process was maintained such that a reasonable number of operators were required. The processes were designed to run twice a week with 94 batches/yr.

Both purification processes were designed using typical bioseparation operations. Some issues, such as the removal of endotoxins and of endogenous DNA, were not specifically addressed during the process design. The validation studies that would be required by the FDA to ensure adequate removal of endotoxin or endogenous DNA were beyond the scope of this economic comparison.

Conventional Purification

The conventional process started with heat sterilization of the buffer components and glucose at 122°C (Fig. 1A). The trace salt solution was filter sterilized and mixed with the other media components. The fermentation took 38 h at 37°C, with ammonia being fed to maintain the pH during the fermentation. The final cell density was 50 g of dry cells/L. The recombinant protein product (GRP) was assumed to accumulate to a final concentration of 0.05 g/g of biomass, or approx 10% of the total cellular proteins. The fermentation broth was harvested and cooled to 4°C before further processing, which ended the fermentation section. The cell broth was first passed through a microfilter to recover the cells and then resuspended in a phosphate buffer. The cell broth stream was then sent to a high-pressure homogenizer for cell lysis. The broth underwent two passes with a pressure

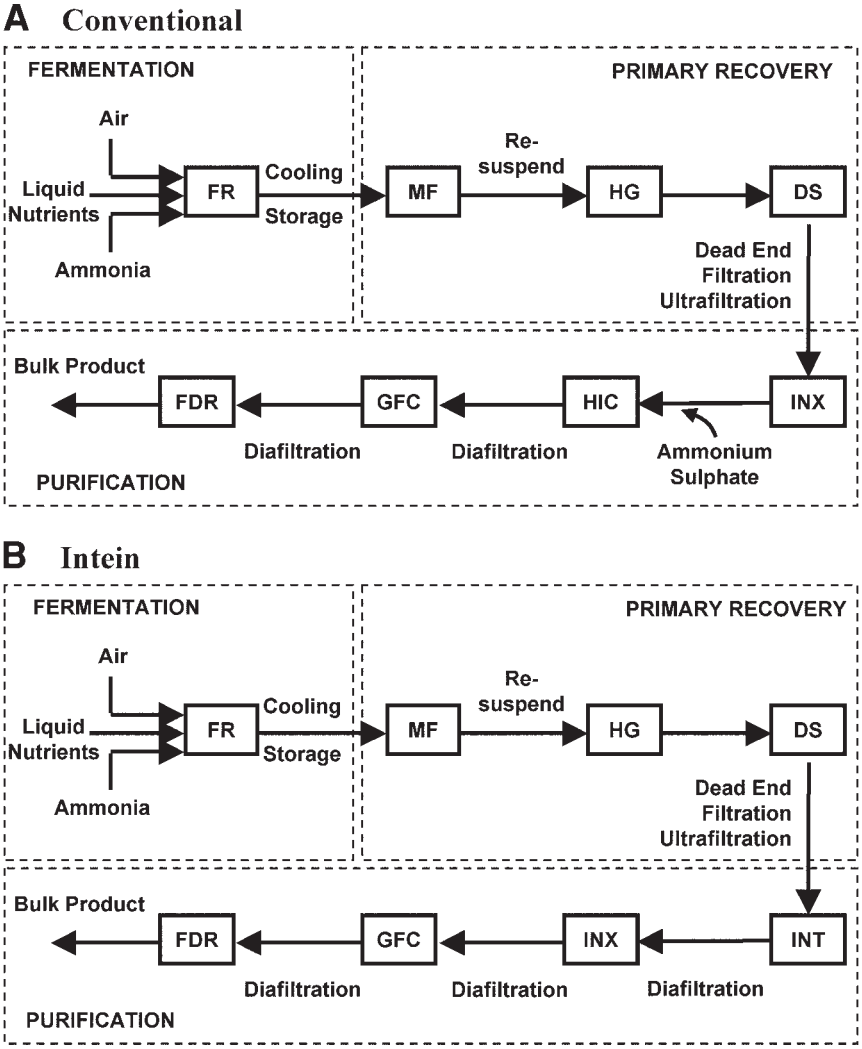

Fig. 1. Block diagram for production of a 25-kDa generic recombinant protein: **(A)** conventional process; **(B)** intein process. FR, Fermentation; MF, microfiltration; HG, homogenization; DS, disk-stack centrifugation; INX, ion-exchange chromatography; INT, intein purification; HIC, hydrophobic interaction chromatography; GFC, gel filtration chromatography; FDR, freeze drying.

drop of 800 bar. Cell debris was separated from the soluble cell extract in a disk-stack centrifuge. The soluble cell extract was filtered by a dead-end filtration device to ensure complete removal of all cell debris and then concentrated by ultrafiltration. The ultrafilter was the last piece of equipment in the primary recovery section.

The purification section started with the ion-exchange chromatography step that was used to separate nucleic acids and numerous host proteins from the target protein by differences in charge. The GRP was eluted

by applying an NaCl gradient (0.05–0.3 *M* in a phosphate buffer) to the column. The ion-exchange column was prepared for the next cycle by washing with NaCl (1 *M*), water for injection (WFI), NaOH (0.5 *M*), and WFI. This column was then equilibrated with a phosphate buffer. The eluate obtained from the ion-exchange column was mixed with a 2 *M* ammonium sulfate solution to a final concentration of 1 *M* in a blending tank and fed to the HIC column. Gradient elution (1–0 *M* ammonium sulfate) was carried out to recover the target protein. The column was prepared for reuse by an isopropyl alcohol (30% [v/v]) strip step followed by regeneration with NaOH (0.5 *M*) and equilibration with a high-salt (1 *M* ammonium sulfate) phosphate buffer. The eluate from the HIC step was dialyzed to remove salts and fed to the gel filtration column for final polishing. An NaCl solution (0.15 *M* in phosphate buffer) was used as the eluent. Before the next cycle, the column was washed with WFI and equilibrated with phosphate buffer. Another diafiltration step following gel permeation chromatography removed the salts and concentrated the eluate, which contained the product with only minimal impurities. Freeze drying was the final purification step, which produced the bulk product.

Intein Process

The primary recovery and fermentation sections of the intein process were similar to those for the conventional process (Fig. 1B). A higher quantity of biomass was required to produce the same amount of product, because the specific production of the target was lower on a per-cell basis. Thus, the fermentation volume for the intein process was significantly larger. The primary recovery section started with microfiltration and resuspension of the cells in a phosphate buffer containing NaCl (0.5 *M*) and EDTA (1 m*M*). The cell broth was homogenized by high pressure. A disk-stack centrifuge was used to remove cell debris. The cell supernatant was passed through a dead-end filter to remove all remaining cell debris and concentrated by ultrafiltration.

The concentrated supernatant was loaded onto an expanded-bed chitin chromatography column. The chitin resin selectively adsorbed the GRP-intein-CBD fusion protein from the feed. Most host proteins and nucleic acids passed through the column, and additional contaminants were removed from the column by a phosphate buffer wash step. Cleavage of GRP from the intein-CBD fusion protein was induced by a cleavage buffer—a phosphate buffer containing dithiothreitol (50 m*M* DTT). The cleavage buffer was passed through the column, followed by a 16-h incubation step to allow the cleavage reaction to occur. Cleaved GRP was eluted from the column with phosphate buffer. The cleavage efficiency was set to 60%; however, cleavage efficiencies as high as 90% have been observed experimentally *(28)*. Small amounts of host proteins and nucleic acids have been experimentally observed to coelute with the target protein; therefore, host protein and nucleic acid contaminants were simulated to coelute from the expanded-bed chitin column. To prepare for reuse, the chitin column

Table 1
Raw Material Costs (2004 Prices)

Raw material	Unit cost ($/kg)
Media buffer salts	2.96
Media trace metal salts	5.92
Ammonia	0.83
NaCl	1.46
Sodium Citrate	2.96
NaOH	4.14
Tris-HCl	7.10
DTT	2150.00
Sodium phosphate monobasic	2.07
Sodium phosphate dibasic	1.77
Sodium phosphate dibasic dihydrate	1.33
Isopropanol	3.86
Ammonium sulfate	0.30
Phosphoric acid	2.66
EDTA	21.90
WFI	0.10
Water	0.01

was regenerated with NaOH (0.3 *M*) and washed with water before equilibration with a phosphate buffer. The chitin purification step was followed by diafiltration to remove salts. An ion-exchange column was used to separate remaining host proteins and nucleic acids from the target protein. Operation of the ion-exchange column was similar to that used for the conventional process. The eluate from the ion-exchange column was dialyzed to reduce the salt content of the stream. A size-exclusion chromatography column was then used as the final polishing step. Operation of the size-exclusion column was identical to that in the conventional purification process. The eluate from the size-exclusion column was dialyzed against water to remove salts. Freeze drying was the final purification step, which produced the bulk product.

Economic Analysis

Raw material costs for most components were based on estimates from Harrison et al. *(29)* using cost indices. Some raw material costs were estimated from supplier list prices for the largest quantity available. SuperPro estimated equipment and consumables costs based on capacity and requirements from data stored in its internal database. Table 1 provides the raw material prices used. The chitin resin cost was assumed to be equal to that of all other resins ($200/L). The cost of waste disposal was set between $0.1/kg and $0.0001/kg, depending on the concentration and nature of the waste materials in the stream. The cost of electricity was assumed to be $0.07/kWh. Capital costs were determined by SuperPro based on factors commonly employed in bioprocessing plants *(29)*.

The total fixed investment was calculated as a sum of the direct fixed capital, working capital, and start-up costs. The year of analysis was 2004. SuperPro used cost indices to account for inflation. Straight-line depreciation was used over a period of 10 yr with a salvage value equal to 5% of the initial direct fixed capital. To compare the cost of production between the two processes, a product cost ($/kg) was calculated. The product cost was based on the annual revenue ($/yr) required to obtain a uniform series disbursement for the total capital investment with a 20% internal rate of return (IRR) over a 15-yr period. Income tax was assumed to be 40%. The plant construction period was 2.5 yr, with a start-up time of 6 mo. The plant was set to operate at 75% of its capacity during the first year and 100% thereafter.

Results and Discussion

Simulation Results

Conventional Process

The overall yield for the conventional process was 40%. The conventional process recovered 1.07 kg of GRP per batch. The total batch time was 91 h, with a minimum cycle time of 81 h. Ninety-four batches were run annually, resulting in a production rate of 100 kg/yr. The fermentation step was the most time-consuming operation in this process. Figure 2A shows the volumetric output through the major equipment in the conventional process. The yields of GRP for each major process step are also shown in Fig. 2A. The product cost for the conventional process was $151/g of GRP.

Intein Process

The overall yield of the intein purification process was approx 30%, which is lower than that for the conventional process. The individual major equipment capacities had to be larger for the intein process to achieve the same annual production owing to the lower cellular productivity and process yield. The total batch time for the intein process was 111 h, with a minimum cycle time of 81 h. Again, by design, 94 batches were run annually with a production rate of 100 kg/yr. The fermentation step was also the most time-consuming operation in the process. Figure 2B shows the volumetric output of the major equipment in the intein process. The product cost for the intein process was $308/g of GRP.

Explanation of Differences Between the Two Processes

The difference in overall yields between the two processes can be attributed to a lower yield from the chitin column. All other operating and equipment parameters were similar. The lower yield from the chitin step was owing to incomplete cleavage of the immobilized fusion protein. The intein process had a longer batch time owing to the intein purification step, which included a 16-h incubation step. Additionally, the primary recovery steps took longer for the intein process owing to the higher fermentation

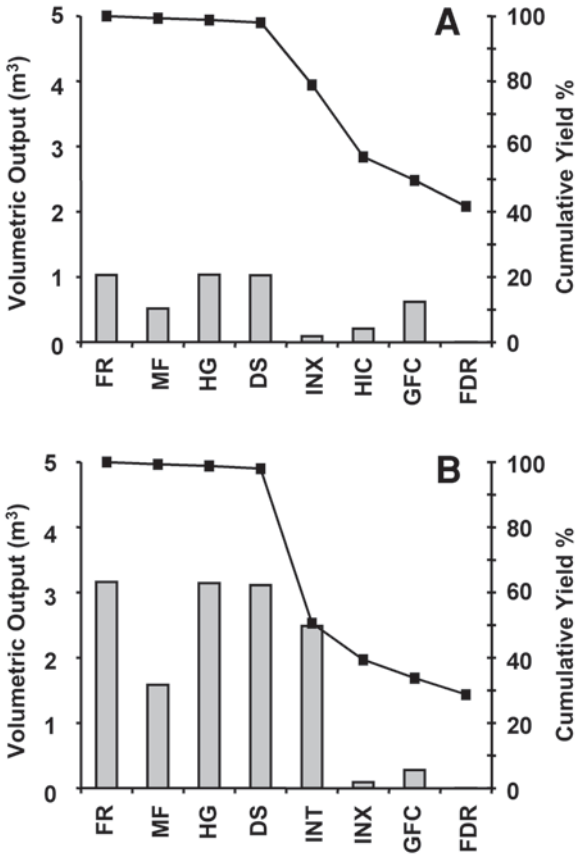

Fig. 2. Volumetric throughput (bars) and target protein yield (line) for **(A)** conventional purification and **(B)** intein purification processes. Abbreviations are the same as for Fig. 1.

volume. The minimum cycle times were equal for the two processes such that the number of batches run per year were equal.

Total Capital Investment

Conventional Process

SuperPro estimated the total capital investment for the conventional plant to be $25 million. The direct fixed capital was $23 million. The direct fixed capital included direct and indirect plant costs associated with the construction of the plant, contractor's fee, and contingency expenses. Direct plant costs included equipment purchase costs and the setup of all supporting facilities required to run the plant. The direct plant costs amounted to $12.9 million. Table 2 provides the costs for major equipment. The indirect plant costs (engineering and construction costs), contractor's fee, and contingency expenses were $10.1 million. In addition to the direct fixed capital,

Table 2
Major Equipment Costs for Conventional Process

Equipment	Units	Total size/capacity	Total cost ($)
Nutrient vessels	2	1.13 m^3	250,000
Heat sterilizer	1	0.30 m^3/h	122,000
Dead-end filter	2	0.63 m^2	36,000
Air compressor	1	5.24 kW	53,000
Air filters	2	0.02 m^3	19,000
Fermentor	1	1.26 m^3	487,000
Blending and storage vessels	3	2.5 m^3	276,000
Microfilter	1	6.43 m^2	22,000
Homogenizer	1	0.26 m^3/h	17,000
Disk-stack centrifuge	1	σ = 11,000 m^2	80,000
Ultrafiltration	1	5.0 m^2	21,000
Ion-exchange column	1	0.30 m^3	152,000
Gel-filtration column	4	1.02 m^3	988,000
Hydrophobic interaction column	1	0.10 m^3	153,000
Diafiltration	2	17.9 m^2	60,000
Freeze dryer	1	0.09 m^2 [a]	425,000

[a]The amount of water was 75 kg/cycle.

the total capital investment included working capital and start-up costs, which were $1.2 million and $386,000, respectively.

Intein Process

The total capital investment for the intein purification process was $29 million. The total direct plant costs were $14.5 million. Table 3 provides the major equipment purchase costs. The indirect plant costs, including the contractor's fee and contingency expenses related to the plant, were $12.2 million. Thus, the direct fixed capital was estimated to be $26.7 million. The working capital and start-up costs, estimated to be $1 million and $1.3 million, respectively, accounted for the remaining total capital investment.

Explanation of Differences Between the Two Processes

The differences in the total capital investment were mainly owing to equipment costs. The intein purification process required larger-capacity equipment owing to the larger fermentation volumes. Lower yields in the chitin purification step and low binding capacity of the chitin resin, compared to traditional ion-exchange chromatography, also increased the required process capacity. Other direct plant costs, as well as the indirect plant costs, were calculated as a fraction of the equipment purchase costs. Thus, the final total capital investment was larger for the intein process compared with the conventional process.

Table 3
Major Equipment Costs for Intein Process

Equipment	Quantity	Total size/capacity	Total cost ($)
Nutrient vessels	2	3.45 m^3	287,000
Heat sterilizer	1	0.94 m^3/h	173,000
Dead-end filter	2	1.91 m^2	36,000
Air compressor	1	16.1 kW	53,000
Air filter	2	0.15 m^3/s	19,000
Fermentor	1	3.89 m^3	570,000
Blending and storage tanks	2	7.0 m^3	191,000
Microfilter	1	19.8 m^2	42,000
Homogenizer	1	0.79 m^3/h	20,000
Disk-stack centrifuge	1	σ = 33,000 m^2	146,000
Ultrafilter	1	15.2 m^2	40,000
PBA chromatography column	1	0.09 m^3	100,000
GFL chromatography column	2	0.92 m^3	454,000
EBA chromatography column	4	1.61 m^3	1,084,000
Diafiltration units	3	71.1 m^3	134,000
Freeze dryer	1	0.08 m^2 [a]	205,000

[a]The amount of water was 33.4 kg/cycle.

Operating and Raw Material Costs

Conventional Process

The annual operating costs for the conventional purification process were $9.9 million. Figure 3A provides a breakdown of these costs. The equipment-dependent cost, which included depreciation, maintenance, and insurance, was 44%, the largest fraction of the annual operating costs. The costs of labor (25%), raw materials (17%), and consumables (9%) were the other major portions of the operating costs. Figure 4A provides a breakdown of the raw material costs. The annual raw material costs were $1.7 million. The cost of water was 62% of all raw material costs. WFI, used in the purification section, accounted for 28% of raw material costs. Other components, including media and buffer components, accounted for the remaining 10%.

Intein Process

The annual operating costs for the intein process were $24.6 million. Figure 3B provides a breakdown of these costs. The largest fraction of the annual operating costs was raw materials, which accounted for nearly 36% of the operating costs. Consumables (32%), equipment-dependent cost (20%), and labor (10%) were the other major fractions of the annual operating costs. Figure 4B provides a breakdown of the raw material costs. The annual raw material cost for the intein process was $8.8 million. DTT, used in the cleavage buffer of the intein purification step, was the largest portion,

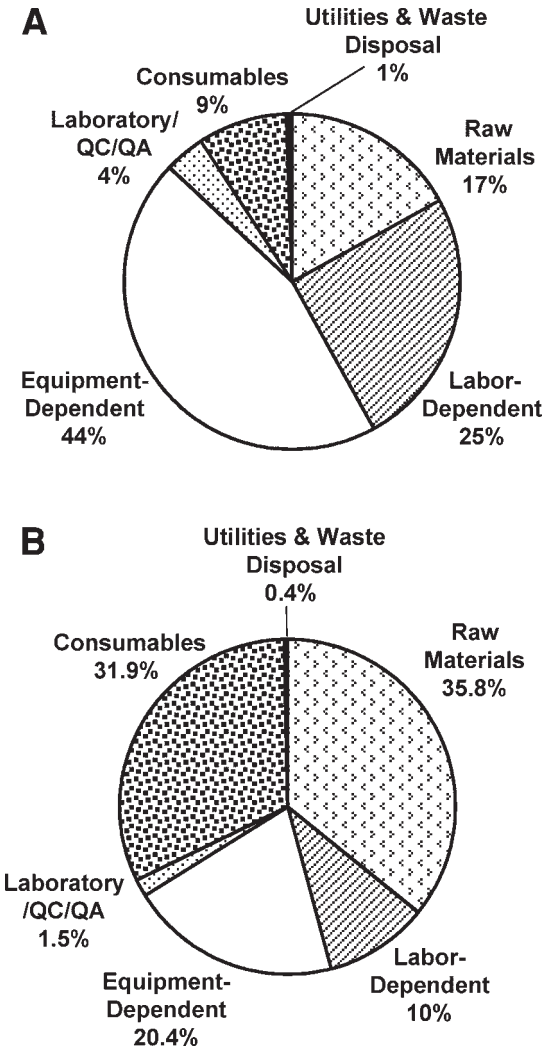

Fig. 3. Breakdown of annual operating costs for **(A)** conventional and **(B)** intein processes. Total annual operating expenditures were $9.9 million and $24.6 million for the conventional and intein processes, respectively. QA, quality assurance; QC, quality control.

at 52%. Water and WFI costs were also significant, at 36 and 7%, respectively. Buffer and media components accounted for the remaining 5% of the raw material cost.

The higher cost of raw materials and consumables resulted in higher operating costs for the intein process. Higher equipment cost also resulted in higher equipment-dependent cost for the intein process; however, these costs impacted the product cost less than the raw material cost. The high raw materials cost for the intein process was attributed to the need for a larger process capacity to achieve the same production level as the conven-

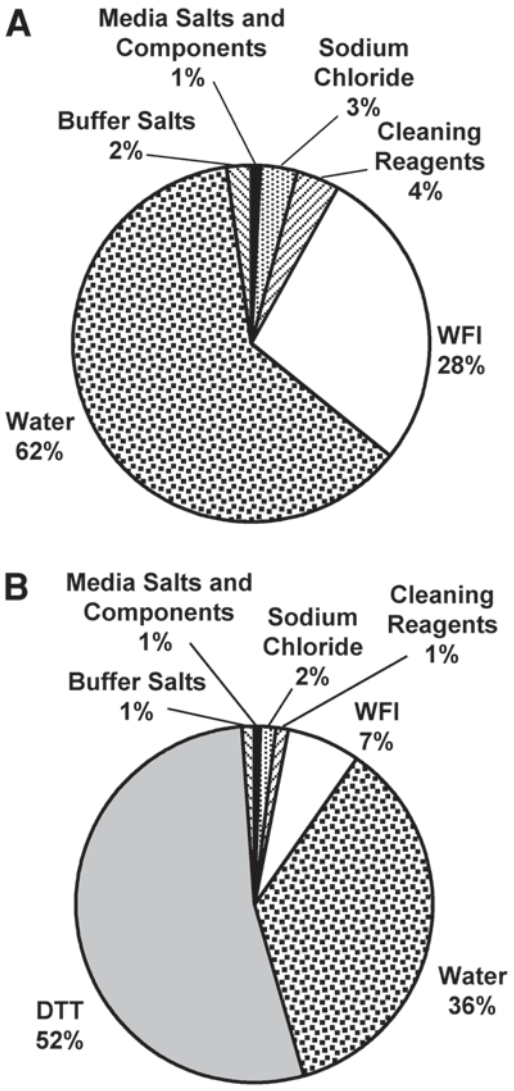

Fig. 4. Breakdown of annual raw material costs for **(A)** conventional and **(B)** intein processes. Total annual raw material costs were $1.7 million and $8.8 million for the conventional and intein processes, respectively.

tional process. Another significant cost for the intein process was the DTT cost, at more than $2000/kg. Water was used in both processes for cleaning and in the fermentation media. WFI was used in buffers for the purification sections of both processes and for removal of salt in the diafiltration steps. The cost of consumables was greater for the intein process, mainly owing to the large quantity of chitin resin required. The cost of the chitin resin was set to $200/L, equal to that of the ion-exchange resin. This issue of chitin resin cost is addressed in greater detail under Sensitivity Analysis.

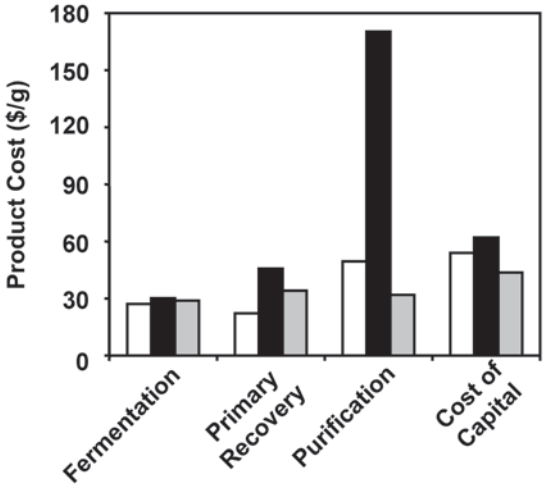

Fig. 5. Operating cost distribution and cost of capital for production of a generic recombinant protein for conventional (□), intein (■), and optimized intein (▩) processes.

Product Cost Distribution

The product cost distribution by section is shown in Fig. 5 for both processes. The cost of capital was defined as the annual uniform series cash receipt on the total capital investment for a 20% IRR over a period of 15 yr/kg of product. As expected, the majority of the operating costs for the conventional and intein processes was attributed to the purification section. A reduction in the operating costs related to the purification section of the intein process would have the greatest impact on product cost. Four process-related factors that increased the product cost for the intein process compared with the conventional process were as follows:

1. Higher process volume owing to fusion protein constraint.
2. Low protein-binding capacity of the chitin resin.
3. Lower recovery from the intein step.
4. Higher raw material cost.

A sensitivity analysis was conducted to determine the effect of these factors on the overall product cost. The results are discussed next.

Sensitivity Analysis

Parallel conventional and intein process simulations indicated that the intein-mediated purification would be significantly more expensive than the conventional purification process for a generic recombinant protein. These simulation results also allowed the identification of critical steps in the process that significantly impacted product cost. The objective of the sensitivity analysis was to determine the effect of potential process improvements on the product cost for the intein system and, where appropriate, for the conventional process. Based on the four factors that signifi-

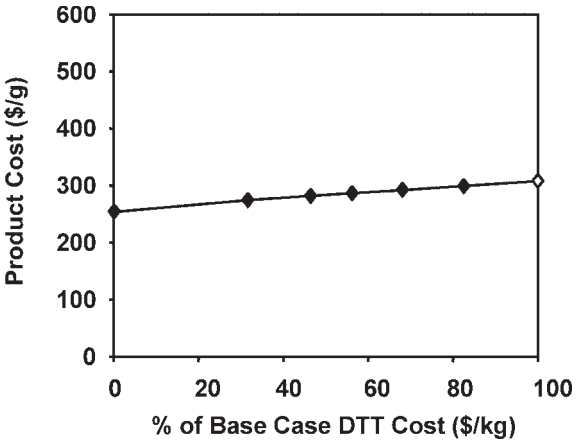

Fig. 6. Effect of DTT cost on product cost for the intein process. The open symbol represents the base case.

cantly affected the product cost for the intein process, five process assumptions that further research might impact were identified. Additionally, process assumptions that would affect either the intein or both processes were identified. The sensitivity analysis examined these nine factors:

1. Cleavage buffer cost.
2. Cleavage efficiency.
3. Chitin resin binding capacity.
4. Chitin resin reusability.
5. Target protein size.
6. Annual process capacity.
7. Expression level of recombinant protein.
8. Expanded bed adsorption.
9. Use of Tris buffers.

Based on the sensitivity analysis, an optimized intein process was designed.

Cleavage Buffer Cost

The most expensive raw material was DTT, which accounted for 52% of all raw material costs for the intein process. DTT was a component of the cleavage buffer that induced cleavage of the intein fusion protein via a reduction reaction *(8)*. If one assumes a lower cost for DTT or replacement of DTT by a less expensive reducing agent, it is expected that product cost would be significantly reduced for the intein process. The sensitivity analysis to find the effect of DTT cost on product cost was conducted by varying the chemical cost by a power law, $Y_1 = Y_0(X_1/X_0)^n$. X_i was the quantity of the raw material used in the process and Y_i was the corresponding cost *(30,31)*. The value of n was varied from 1 (for the base case) to 0.85 to simulate cheaper DTT. As shown in Fig. 6, the product cost decreased almost linearly

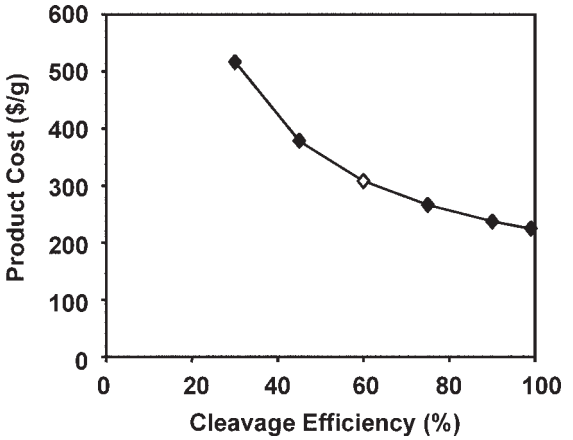

Fig. 7. Effect of cleavage efficiency on product cost for the intein process. The open symbol represents the base intein process.

with a decrease in the DTT cost. At 30% of the base-case DTT cost, the product cost was reduced by about 10%. The magnitude of this change was owing to the large fraction that DTT cost contributed to the operating costs. Thus, alternative supplies of DTT, alternative sources of reducing agent, or other methods to induce cleavage could significantly reduce product cost. One alternative cleavage method that has been described uses changes in temperature and pH to induce the cleavage reaction without DTT *(10,12)*. This particular cleavage system was simulated by reducing the cost of DTT to the cost of a standard buffer salt. This assumption reduced the product cost by 17% with respect to the intein base case to $254/g of GRP.

Cleavage Efficiency

Cleavage efficiency depends on the particular intein protein selected, the amino acid residues at the junction of the intein and flanking proteins, and the fusion protein conformation. Cleavage efficiency is also affected by the mode of cleavage (change in pH and temperature vs DTT). The cleavage efficiency directly affected process yields and product cost by determining recovery at the intein step. The sensitivity of the product cost to the cleavage efficiency in the intein process was determined by varying the cleavage efficiency from 30 to 99%, where the base case was 60%. In Fig. 7, the sensitivity of the product cost to the cleavage efficiency is shown. The product cost increased significantly as the cleavage efficiency decreased. At a cleavage efficiency of 30%, the product cost was more than one and a half times that of the intein base case. A cleavage efficiency of 90% resulted in a 23% reduction in the product cost, to $237/g. Note that the cleavage efficiency affected both the capital investment and operating costs by changing product yield. Because the cleavage efficiency depends on the aforementioned factors, it may not always be possible to modify the cleavage efficiency for a particular target protein. Thus, a protein with extremely low cleavage

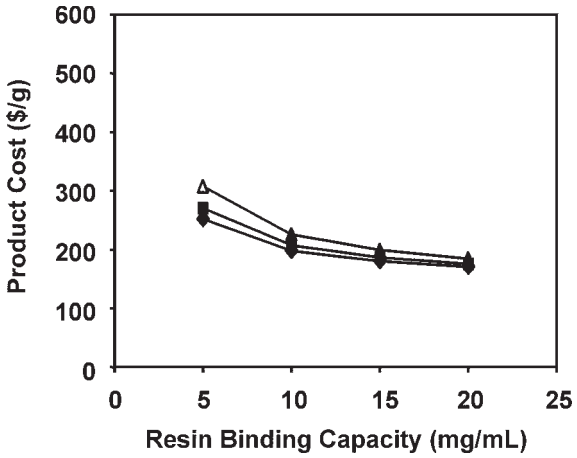

Fig. 8. Effect of chitin resin binding capacity on product cost for the intein process. The cost of the resin was set at \$50/L (◆), \$100/L (■), and \$200/L (▲). The open symbol represents the base intein process.

efficiency may not be economically produced by an intein process, whereas a target protein with a high cleavage efficiency may be produced economically by an intein process.

Chitin Resin Binding Capacity

The experimentally observed chitin resin binding capacity during laboratory-scale purifications was 5 mg/mL, which is low compared with other protein purification resins. This binding capacity assumption significantly increased the number of chitin columns required for the intein step compared with a conventional ion-exchange step. Additionally, the low binding capacity increased the cost of consumables. Since chitin resins have not been extensively studied in purification columns, it was hypothesized that research to improve chitin resin properties should be able to address this issue *(32,33)*. Therefore, the product cost sensitivity to the binding capacity was investigated. Figure 8 presents the results of the simulations. As expected, increased binding capacity decreased the product cost. Doubling the binding capacity to 10 mg/mL reduced the product cost about 25%, to \$226/g. This reduction in product cost was attributed to a direct reduction in the cost of consumables and a decrease in the cost of equipment. Furthermore, it was observed that as the chitin-binding capacity increased, the product cost became less sensitive to the chitin resin cost. For example, if the binding capacity were increased to 20 mg/mL, the product cost would decrease 37%, to approx \$194/g, even if the chitin resin was assumed to cost \$300/L (50% more than the base case). This suggested that further research into chitin bead resins would potentially benefit the intein process, even if the resin cost increased.

The data for chitin resin binding capacity were obtained from laboratory-scale experiments. These laboratory columns were operated under

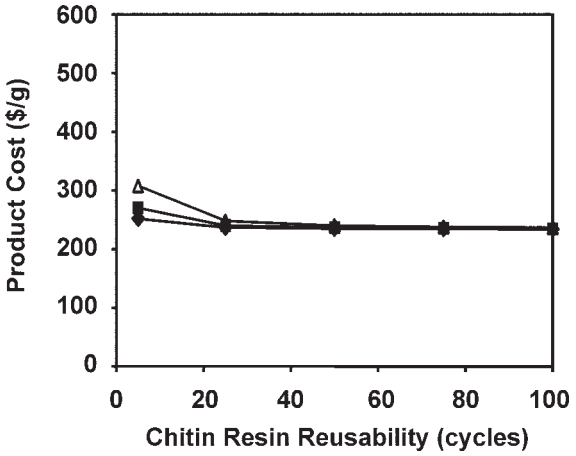

Fig. 9. Effect of chitin resin reusability on product cost for the intein process. The cost of the resin was set at \$50/L (◆), \$100/L (■), and \$200/L (▲). The open symbol represents the base intein process.

gravity flow. Under these conditions, it was observed that a fluorescent-labeled intein CBD fusion protein had low binding owing to poor flow characteristics in the column (unpublished data). In these simulations, an expanded-bed adsorption column was used to improve flow characteristics; however, without further performance data, the intein base-case simulation used the low (5 mg/mL) binding capacity estimated from laboratory studies. Recent efforts have been made toward the preparation of an improved chitin resin. These involve using a glass support for strength and vortex flow for better contact between the fusion protein and the resin *(34)*.

Chitin Resin Reusability

The currently available chitin resin has limited reusability (five cycles), in part owing to the fragile nature of the chitin beads. This reusability is significantly lower than that of commercial protein purification resins. The sensitivity of the product cost to the chitin resin was examined. For the base-case binding capacity, when the reusability was increased, the product cost decreased (Fig. 9). This effect was significant only up to a reusability of 25 cycles. For example, for the base-case resin cost (\$200/L), when the reusability was simulated to be 25 cycles, the product cost was \$248/g and only increased marginally, to \$255/g, when the resin cost was increased to \$300/L with the same reusability. The product cost was not as sensitive to the reusability as it was to the binding capacity. Increasing the reusability of the chitin resin to at least 25 cycles would positively impact the economics of the intein process.

Currently, chitin resins are produced using a solution of chitosan, which is treated with acetic anhydride. This process acetylates the chitosan, converting it into chitin. Chitin has low solubility in aqueous solvents, which results in the chitin forming colloidal particles (the chitin resin) *(8,32)*.

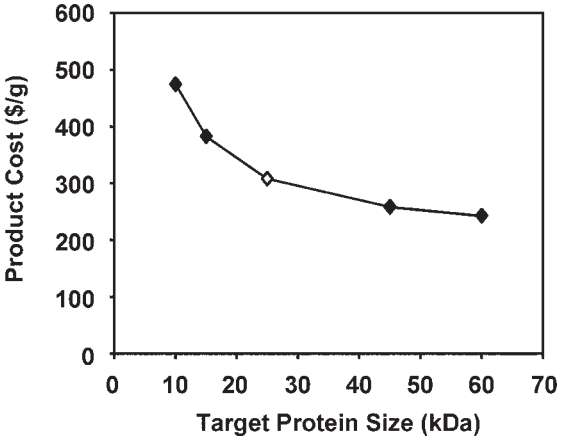

Fig. 10. Effect of target protein size on product cost for the intein process. The open symbol represents the base intein process.

The raw material, chitosan, is obtained from natural waste sources, such as crab, shrimp, and lobster shells. Because the chitin resin production method is fairly simple, further research should improve production techniques such that a chitin resin with acceptable physical and chemical properties can be manufactured.

Target Protein Size

A significant difference between the two simulated processes was the greater biomass required to maintain the intein process production capacity equal to that of the conventional process. This difference was owing to the assumption that cells have a fixed capacity to express a recombinant protein (grams of recombinant protein per cell). In the conventional process, the cells expressed only the GRP; in the intein process, the GRP-intein-CBD fusion protein was expressed, only a fraction of which is the target protein. To determine the effect of target protein size on product cost, the intein process was simulated with different target protein sizes (10–60 kDa). Given the assumption of fixed expression capacity for the cell, this effectively modeled a change in the mass ratio of the target protein to the intein-CBD tag. This assumption resulted in a decrease in product cost as the target protein size increased relative to the intein CBD tag. Figure 10 presents the target size simulation results. The product cost for a 10-kDa target protein was nearly double the product cost of a 60-kDa protein. Thus, the intein process would be better suited for the purification of large proteins relative to the intein fusion tag. Smaller intein fusion tags or larger target proteins would result in the same economic benefits.

Annual Process Capacity

Current market sizes for recombinant proteins vary from a few kilograms (for erythropoietin) to about 2000 kg/yr (for insulin) *(19)*. Some

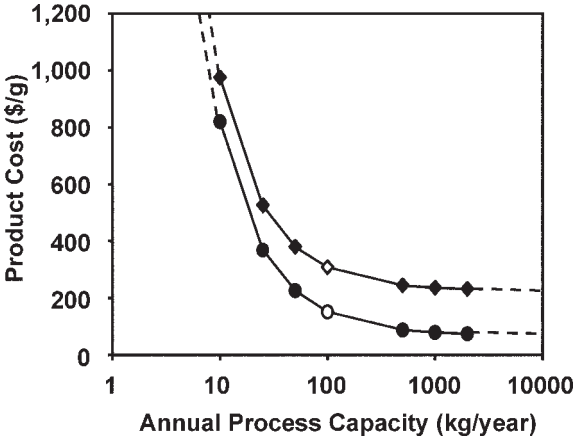

Fig. 11. Effect of annual capacity on product cost for the conventional (●) and intein (◆) processes. Open symbols represent the base cases.

potential therapeutics, such as α-1-antitrypsin, have estimated US markets of about 9000 kg/yr *(35,36)*. Therefore, the effect of annual production capacity on product cost was investigated. The annual production capacity for the base-case simulations was 100 kg/yr of product. For the intein and conventional processes, the effect of process size on process economics was examined. These simulation results indicated that both processes were very sensitive to the annual production rate (Fig. 11). Product costs leveled off for processes larger than 1000 kg/yr, as shown in Fig. 11. These results were expected, because equipment would be more efficiently utilized at higher production rates.

Expression Level of Recombinant Protein

Because it is well known that recombinant protein expression levels can vary from trace amounts to very high quantities, the effect of expression level on product cost was investigated for both the intein and conventional processes. The expression level of the target protein determined the amount of biomass required to meet the annual production rate; that is, higher expression levels lowered the biomass requirements. The biomass requirements directly determined equipment sizes. To determine the effect of expression level on the economics of the two processes, the recombinant protein content in the cells was varied from 25 to 100 mg/g of dry cell weight (50 mg/g of dry cell weight was the base case). The simulation results demonstrated that the process economics were not very sensitive to an increase in the recombinant protein expression level beyond the base case, as shown in Fig. 12. The reason that the expression level did not affect production cost to a great extent was that the purification section costs dominated. For processes with lower purification cost, it is expected that the expression level would affect product cost to a greater extent. Although

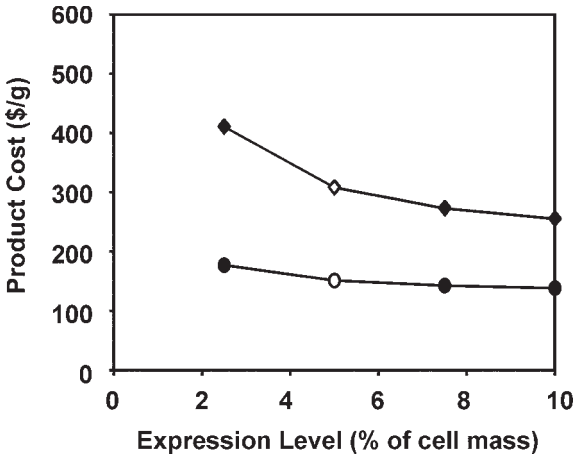

Fig. 12. Effect of recombinant protein expression level on product cost for the conventional (●) and intein (◆) processes. Open symbols represent the base cases.

an increase in the expression level decreased the quantity of biomass to be processed, it did not greatly decrease the load on the purification section of the process. Thus, doubling the expression level resulted in only an 18% decrease in the final product cost for the intein process. The conventional process was even less sensitive to the expression level, and doubling the expression level decreased the product cost by only 9%.

Expanded-Bed Adsorption

The use of expanded-bed adsorption is a fairly new unit operation *(29)*. In the intein process base case, conservative estimates were used to design the cell and cell debris removal steps. Cell debris was removed by a disk-stack centrifuge, dead-end filtration, and ultrafiltration prior to the expanded-bed chitin chromatography adsorption step. Some reports have indicated that expanded beds are capable of handling cell debris during the loading step without compromising performance *(29,37–42)*. Two alternative processes were thus simulated for removal of cell debris. The first alternative removed the ultrafiltration step. The second alternative removed both the ultrafiltration and dead-end filtration steps. Surprisingly, both alternative processes were more expensive than the intein base case, with product costs of $366/g and $369/g, respectively. The main cause for the increased costs was the additional host protein. In the two alternative process simulations, the percentage of binding of impurities to the chitin resin was the same as for the base case. The removal of the dead-end filtration and ultrafiltration steps increased the total quantity and the relative concentration of the impurities with respect to the GRP-intein fusion protein. Thus, a significant amount of chitin resin was required to capture the impurities, which negatively impacted the product cost. When the amount of impurities bound to the chitin resin was held constant,

instead of the percentage of binding, the product cost was unchanged by the elimination of the ultrafiltration and dead-end filtration steps. The product cost was unchanged owing to additional storage requirements to maintain the proper flow in the process in the absence of the two filtration steps. The alternative simulation results do not globally indicate that the use of expanded-bed adsorption immediately following cell disruption will always be economically ineffective; however, the results indicate that further characterization of the chitin resin with respect to impurity adsorption is needed.

Tris Buffers

Many laboratory processes routinely use Tris buffers, which are relatively expensive buffering agents. For this reason, phosphate buffers were used throughout the intein base-case simulation, even though no experimental data were available for completely phosphate-buffered systems; however, experimental data on the intein cleavage step indicate that phosphate buffers work equally well as Tris buffers. When phosphate buffers were replaced with Tris buffers for the cell resuspension, expanded-bed adsorption chromatography, and ion-exchange chromatography steps, the product cost was not significantly changed, in part owing to the relatively small volumes needed for these steps.

Optimized Intein Process

Overall, the sensitivity analysis demonstrated that the intein process product cost was sensitive to many of the process assumptions, mainly the DTT cost, cleavage efficiency, and binding efficiency of the chitin resin. The cost was also, to a lesser extent, sensitive to the reusability of the chitin resin, recombinant protein expression level, and operation of the expanded-bed chitin column. Based on the sensitivity analysis results, an optimized intein process was simulated. In the optimized intein simulation, the DTT cost was reduced to 30% of the original cost, to \$608/kg. The cleavage efficiency was increased to 90% and resin binding capacity was increased to 20 mg/mL. The chitin resin reusability was increased to 100 cycles. Expression level, process capacity, and chitin resin cost remained the same as for the intein base case (50 mg/g of cell mass, 100 kg/yr, and \$200/L, respectively). As expected, the product cost was drastically reduced. The total capital investment dropped to \$20.4 million and operating costs to \$9.5 million, which were 30 and 61% lower, respectively, than for the intein base case. The optimized intein case had a product cost of only \$138/g, which was about 55% lower than for the intein base case. The optimized intein case product cost was slightly lower than the conventional base case product cost of \$151/g, but not significantly different. The capital distribution for the optimized intein process is presented in Fig. 5. As expected, the major effect that the optimized intein process had on economic feasibility was owing to the significant decrease in purification section costs.

Numerous inteins and affinity fusions have been reported in the literature *(5,8–11,13)*. These simulations investigated the economic feasibility of manufacturing a therapeutic recombinant protein using an intein-mediated purification step based on the IMPACT system. Because none of the intein systems have been used industrially, the sensitivity analysis included optimizations to the process that might be achieved by using a particular intein fusion (e.g., pH-induced cleavage).

The sensitivity analysis showed that with significant improvements in the characteristics of the chitin resin, and development of improved intein cleavage conditions (high efficiency, no DTT), the intein-mediated purification process approached economic feasibility. The conventional process was simulated in parallel to the intein process as a benchmark that incorporated the same material and equipment assumptions. It was expected that the estimated product cost for the conventional process, which provided the benchmark economics, would exceed the current prices for many commonly available drugs. The production of commercially available recombinant protein drugs has been optimized based on experimental data, whereas the GRP simulations presented here did not have this opportunity. Hurdles that were not addressed by our study include acceptance by the FDA and the industrial community.

Conclusion

The intein-mediated protein purification system allows the affinity purification of a recombinant protein using a relatively inexpensive affinity resin. Additionally, the autocatalytic cleavage of the intein-CBD fusion protein from the target protein eliminates the need for a protease and subsequent protease removal step. We investigated the economics of the intein purification process in parallel with a conventional purification process. The simulation results demonstrated that the intein process based on standard laboratory methods was significantly more expensive than the conventional process, mainly owing to the properties of the chitin resin. Sensitivity analysis of the intein process indicated that the process was also very sensitive to the cleavage efficiency and to a lesser extent the DTT cost. Major product cost savings could be realized when the protein binding capacity of the chitin resin was increased, even when these improvements increased the resin cost. The intein process was less sensitive to the reusability of the resin; however, product costs would be decreased if a chitin resin capable of 25 cycles were available.

An optimized intein process was simulated incorporating many of the process changes suggested by the sensitivity analysis—improvement in the chitin resin binding capacity and reusability, improvement in the cleavage efficiency, and reduction in the DTT cost. The optimized intein process was slightly less expensive than the conventional process owing to reduced operating costs in the purification section. Further research is necessary to produce chitin resins with acceptable properties. An intein-mediated

purification process could be economically feasible if improvements to the chitin resin could be implemented.

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