Computer-Aided Process Analysis and Economic Evaluation for Biosynthetic Human Insulin Production—A Case Study

Article *in* Biotechnology and Bioengineering · December 1995

DOI: 10.1002/bit.260480516 · Source: PubMed

3 authors, including:

Demetri Petrides
Intelligen, Inc.
126 PUBLICATIONS **851** CITATIONS

SEE PROFILE

Computer-Aided Process Analysis and Economic Evaluation for Biosynthetic Human Insulin Production – A Case Study

By Demetri Petrides, Elpida Sapidou and John Calandranis
Intelligen, Inc., Scotch Plains, NJ, USA
E-mail: dpetrides@intelligen.com

Introduction

Insulin facilitates the metabolism of carbohydrates and is essential for the supply of energy to the cells of the body. Impaired insulin production leads to the disease diabetes mellitus, which is the third largest cause of death in industrialized countries, after cardiovascular diseases and cancer (Barfoed, 1987). Approximately 18 million people suffer from diabetes in the U.S. (Dalton, 2004). Worldwide, the total number of diabetics is estimated to be between 150 and 200 million (Vadim, 2004) and it is growing at an annual rate of 3-5% (Datar and Rosen, 1990; Petrides et al., 1995).

Human insulin is a polypeptide consisting of 51 amino acids arranged in two chains: A with 21 amino acids, and B consisting of 30 amino acids. The A and B chains are connected by two disulfide bonds. Human insulin has a molecular weight of 5,734 and an isoelectric point of 5.4. Human insulin can be produced by four different methods:

- Extraction from human pancreas
- Chemical synthesis via individual amino acids
- Conversion of pork insulin or "semisynthesis"
- Fermentation of genetically engineered microorganisms

Extraction from the human pancreas cannot be practiced because the availability of raw material is so limited. Total synthesis, while technically feasible, is not economically viable because the yield is very low. Production based on pork insulin, also known as "semisynthesis," transforms the porcine insulin molecule into an exact replica of the human insulin molecule by substituting a single amino acid, threonine, for alanine in the G-30 position. This technology has been developed and implemented by Novo Nordisk A/S (Denmark). However, this option is also quite expensive because it requires the collection and processing of large amounts of porcine pancreases. In addition, its supply is limited by the availability of porcine pancreas.

At least three alternative technologies have been developed for producing human insulin based on fermentation and utilizing recombinant DNA technology (Ladisch and Kohlmann, 1992).

Two-chain method The first successful technique of biosynthetic human insulin (BHI) production based on recombinant DNA technology was the two-chain method. This technique was developed by Genentech, Inc. (South San Francisco) and scaled up by Eli Lilly and Co.

(Indianapolis). Each insulin chain is produced as a β-galactosidase fusion protein in *Escherichia coli,* forming inclusion bodies. The two peptide chains are recovered from the inclusion bodies, purified, and combined to yield human insulin. Later, the β-galactosidase operon was replaced with the tryptophan (Trp) operon, resulting in a substantial yield increase.

Proinsulin method The so-called intracellular method of making proinsulin eliminates the need for the separate fermentation and purification trains required by the two-chain method. Intact proinsulin is produced instead. The proinsulin route has been commercialized by Eli Lilly and Co. (Kehoe, 1989). Figure 1 shows the key transformation steps. The *E. coli* cells overproduce Trp-LE'-Met-proinsulin (Trp-LE'-Met-proinsulin is a 121 amino acid peptide signal sequence; proinsulin, with 82 amino acids, is a precursor to insulin) in the form of inclusion bodies, which are recovered and solubilized. Proinsulin is released by cleaving the methionine linker using CNBr. The proinsulin chain is subjected to a folding process to allow intermolecular disulfide bonds to form; and the C peptide, which connects the A and B chains in proinsulin, is then cleaved with enzymes to yield human insulin. A number of chromatography and membrane filtration steps are utilized to purify the product.

A second method of producing proinsulin was developed by Novo Nordisk A/S. It is based on yeast cells that secrete insulin as a single-chain insulin precursor (Barfoed, 1987). Secretion simplifies product isolation and purification. The precursor contains the correct disulfide bridges and is therefore identical to those of insulin. It is converted to human insulin by transpeptidation in organic solvent in the presence of a threonine ester and trypsin followed by de-esterification. Another advantage of this technology is the ability to reuse the cells by employing a continuous bioreactor-cell separator loop.

In this example, we analyze a process based on the intracellular proinsulin method.

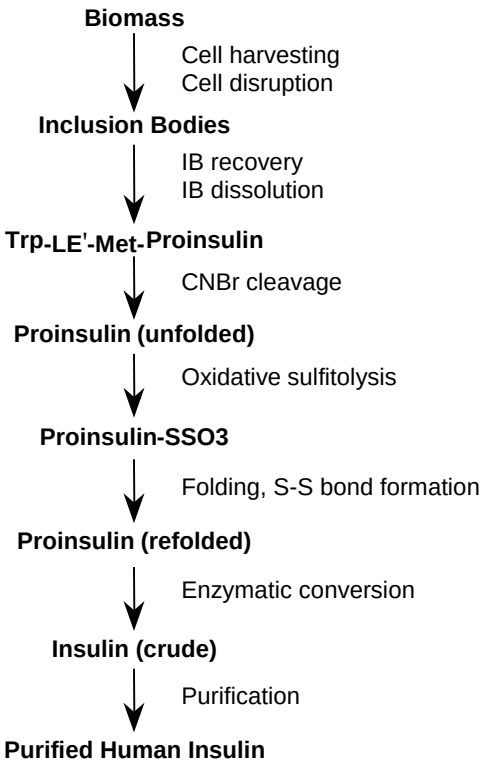

Figure 1 Human insulin from proinsulin fusion protein.

Market Analysis and Design Basis

Treatment with insulin requires on average 0.5 g/patient/year of purified product. Considering the total number of diabetics (150 to 200 million), that corresponds to an annual demand of 75000 to 100000 kg of purified insulin. However, the current worldwide production is only 20000 to 30000 kg/year because most patients in the developing countries cannot afford to pay $250-750/year required for purchasing the medicine. There is a great need for additional capacity and improved processes that can manufacture the product at a lower cost to satisfy the demand in the developing nations. The plant analyzed in this example has a capacity of around 1,800 kg of purified biosynthetic human insulin (BHI) per year. This is a relatively large plant for producing polypeptide-based biopharmaceuticals. It can satisfy the demand of around 3.5 million patients or roughly 25% of the US market. The plant operates around the clock for 330 days a year. A new batch is initiated every 48 hours resulting in 160 batches per year. The fermentation broth volume per batch is approximately 37.5 m^3.

Process Description

The entire flowsheet for the production of BHI is shown in Figure 2. The process was modeled using SuperPro Designer, a batch process simulator from Intelligen, Inc. (Scotch Plains, NJ). It is

divided into four sections: 1) Fermentation, 2) Primary Recovery, 3) Reactions, and 4) Final Purification. A section in SuperPro is simply a set of unit procedures (processing steps).

Fermentation Section Fermentation media are prepared in a stainless steel tank (V-101) and sterilized in a continuous heat sterilizer (ST-101). The axial compressor (G-101) and the absolute filter (AF-101) provide sterile air and ammonia to the fermentor at an average rate of 0.5 VVM. A two-step seed fermentor train (not shown in the flowsheet) is used to inoculate the 50 m^3 production fermentor (V-102) with transformed *E. coli* cells. These cells are used to produce the Trp-LE'-MET-proinsulin precursor of insulin, which is retained in the cellular biomass. The fermentation time in the production fermentor is about 18 hours, and the fermentation temperature is 37 °C. The final concentration of *E. coli* in the production fermentor is about 30 g/liter (dry cell weight). The Trp operon is turned on when the *E. coli* fermentation runs out of tryptophan. The chimeric protein Trp-LE'-MET-proinsulin accumulates intracellularly as insoluble aggregates (inclusion bodies), and this decreases the rate at which the protein is degraded by proteolytic enzymes. In the base case, it was assumed that the inclusion bodies (IBs) constitute 20% of total dry cell mass. At the end of fermentation, the broth is cooled down to 10 °C to minimize cell lysis. After completing each processing step in the Fermentation Section (and subsequent sections), the equipment is cleaned thoroughly in order to prepare for the next batch of product.

-

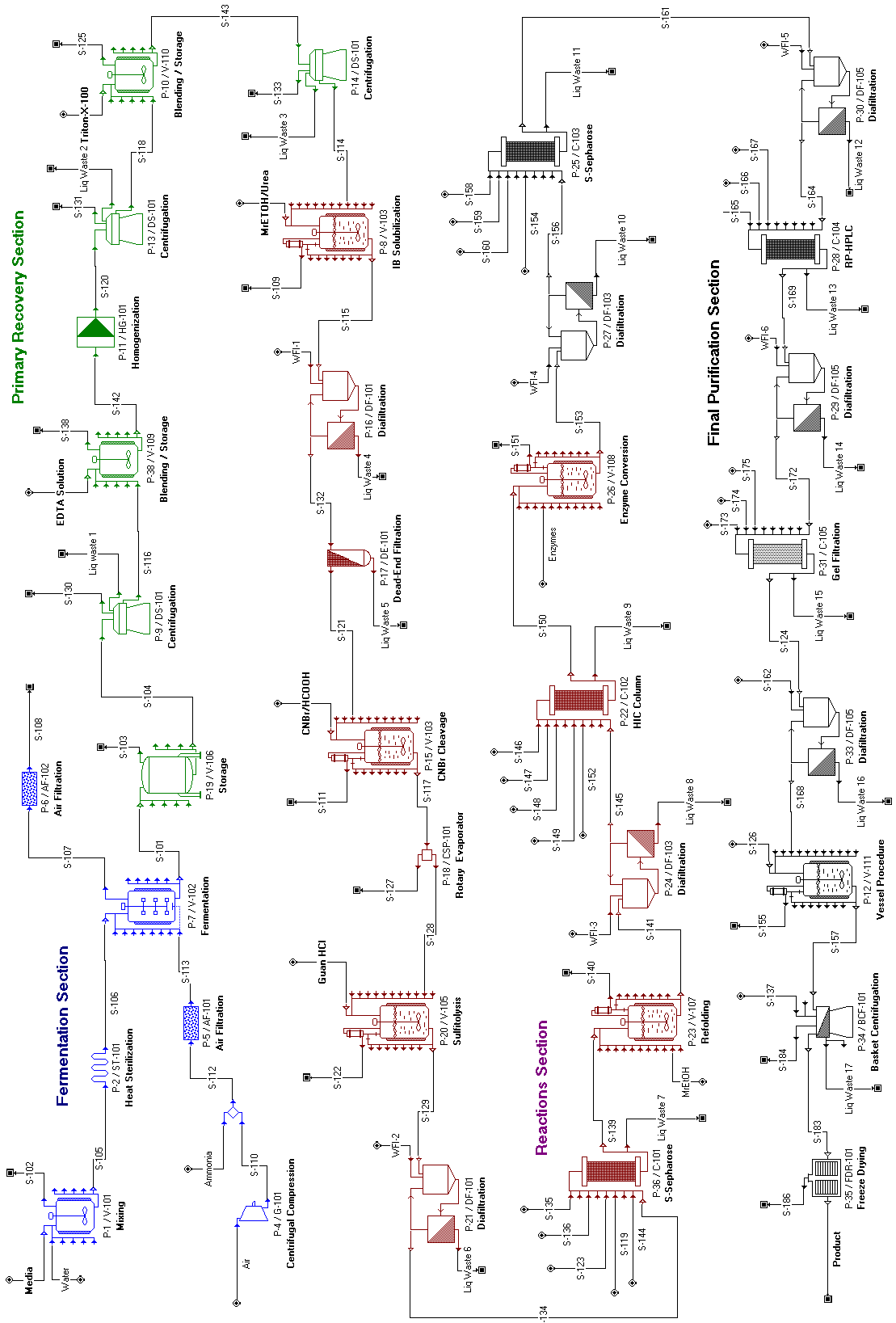

Figure 11-12 Insulin Production Flowsheet

Primary Recovery Section After the end of fermentation, the broth is transferred into a surge tank (V-106), which isolates the upstream from the downstream section of the plant. Three disk-stack centrifuges (DS-101) operating in parallel are used for cell harvesting. Note that a single unit procedure icon on the screen of SuperPro may represent multiple equipment items operating in parallel. During centrifugation, the broth is concentrated from 37000 L to 9165 L, and most of the extracellular impurities are removed. The cell recovery yield is 98%. The cell sludge is diluted with an equal volume of buffer solution (buffer composition: 96.4% w/w WFI (water for injection), 0.7% EDTA, and 2.9% TRIS-base) using a blending tank (V-109). The buffer facilitates the separation of the cell debris particles from inclusion bodies. Next, a high pressure homogenizer (HG-101) is used to break the cells and release the inclusion bodies. The broth undergoes three passes under a pressure drop of 800 bar. The exit temperature is maintained at around 10 °C. The same centrifuges as before (DS-101) are then used for inclusion body recovery (P-13). The reuse of these centrifuges can be seen by noting that procedures P-9 and P-13 have the same equipment name, DS-101. The IBs are recovered in the heavy phase (with a yield of 98%) while most of the cell debris particles remain in the light phase. This is possible because the density (1.3 g/cm^3) and size (diameter about 1 μm) of the IBs are significantly greater than that of the cell debris particles. The IB sludge, which contains approximately 20% solids w/w, is washed with WFI containing 0.66% w/w Triton-X100 detergent (the volume of solution is twice the volume of inclusion body sludge) and recentrifuged (P-14) using the same centrifuges as before (DS-101). The detergent solution facilitates purification (dissociation of debris and soluble proteins from inclusion bodies). The exit temperature is maintained at 10 °C. The slurry volume at the end of the primary recovery section is around 1400 L.

Reactions Section *Inclusion Body Solubilization*. The inclusion body suspension is transferred to a glass-lined reaction tank (V-103) and is mixed with urea and 2-mercaptoethanol to final concentrations of 300 g/L (5 M) and 40 g/L, respectively. Urea is a chaotropic agent that dissolves the denatured protein in the inclusion bodies and 2-mercaptoethanol is a reductant that reduces disulfide bonds. A reaction time of 8 hours is required to reach a solubilization yield of 95%. The inclusion bodies are composed of 80% w/w Trp-LE'-Met-proinsulin, with the remainder being other (contaminant) proteins. At the end of the solubilization reaction, a diafiltration unit (DF-101) is used to replace urea and 2-mercaptoethanol with WFI and to concentrate the solution. This operation is performed in 6 h with a recovery yield of 98%. All remaining fine particles (biomass, debris, and inclusion bodies) are removed using a polishing dead-end filter (DE-101). This polishing filter protects the chromatographic units that are used further downstream. The solution volume at this point is around 5200 L.

CNBr cleavage. The chimeric protein is cleaved with CNBr (cyanogen bromide) into the signal sequence Trp-LE'-Met, which contains 121 amino acids, and the denatured proinsulin (82 amino acids) in the same reactor (V-103) that was used for IB solubilization (procedure P-15). The reaction is carried out in a 70% formic acid solution containing 30-fold molar excess CNBr (stoichiometrically, one mole of CNBr is required per mole of Trp-LE'-Met-proinsulin). The reaction takes 12 h at 20 ^{o}C and reaches a yield of 95%. The mass of the released proinsulin is approximately 30% of the mass of Trp-LE'-Met-proinsulin. A small amount of cyanide gas is formed as a by-product of the cleavage reaction. Detailed information on CNBr cleavage is available in the patent literature (U.S. Patent No. 4,451,396, 1984.). The formic acid, unreacted CNBr, and generated cyanide gas are removed by applying vacuum and raising the temperature to around 35 ^{o}C (the boiling point of CNBr). This operation is carried out in a rotary vacuum evaporator (CSP-101) and takes 1 h. Since cyanide gas is toxic, all air exhausted from the vessels is scrubbed with a solution of hypochlorite, which is prepared and maintained in situ (Kehoe, 1989).

Sulfitolysis. Sulfitolysis of the denatured proinsulin takes place in a reaction tank (V-105) under alkaline conditions (pH 9-11). This operation is designed to unfold proinsulin, break any disulfide bonds, and add SO_3 moieties to all sulfur residues on the cysteines. The product of interest is human proinsulin$(S\text{-}SO_3\text{-})_6$ (protein-S-sulfonate). The sulfitolysis step is necessary for two reasons: (1) the proinsulin probably is not folded in the correct configuration when expressed in *E. coli* as part of a fusion protein, and (2) the cyanogen bromide treatment tends to break existing disulfide bonds. The final sulfitolysis mixture contains 50% w/w guanidine•HCl (6 M), 0.35% ammonium bicarbonate (NH_4HCO_3), 3% Na_2SO_3 and 1.5% $Na_2O_6S_4$ (U.S. Patent No. 4,923,967, 1990). A reaction time of 12 h is required to reach a yield of 95%. The presence of the denaturing reagent (guanidine•HCl) prevents refolding and cross-folding of the same protein

molecule onto itself or two separate protein molecules onto each other. Urea may also be used as a denaturing reagent. Upon completion of the sulfitolysis reaction, the sulfitolysis solution is exchanged with WFI to a final guanidine•HCl concentration of 20% w/w. This procedure, P-21, utilizes the DF-101 diafilter that also handles buffer exchange after IB solubilization. The human proinsulin$(S\text{-}SO_3\text{-})_6$ is then chromatographically purified using three ion-exchange columns (C-101) operating in parallel. Each column has a diameter of 140 cm and a bed height of 25 cm. A cation exchange resin is used (SP Sepharose Fast Flow from GE Healthcare) operating at pH 4.0. The eluant solution contains: 69.5 % w/w WFI, 29% urea, and 1.5% NaCl. Urea, a denaturing agent, is used to prevent incorrect refolding and cross-folding of proinsulin$(S\text{-}SO_3\text{-})_6$. The following operating assumptions were made: (1) the column is equilibrated for 30 minutes prior to loading, (2) the total resin binding capacity is 20 mg/ml, (3) the eluant volume is equal to 5 column volumes (CVs), (4) the total volume of the solutions for column wash, regeneration and storage is 15 CVs, and (5) the protein of interest is recovered in 1.5 CVs of eluant buffer with a recovery yield of 90%.

Refolding. This operation catalyzes the removal of the SO_3^{2-} moiety and then allows disulfide bond formation and correct refolding of the proinsulin to its native form. It takes place in a reaction tank (V-107). This process step involves treatment with mercaptoethanol (MrEtOH), a reductant that facilitates the disulfide interchange reaction. It is added at a ratio of 1.5 mol of mercaptoethanol to 1 mol of SO_3^{2-}. Dilution to a proinsulin$(S\text{-}SO_3)_6$ concentration of less than 1 g/L is required to prevent cross-folding of proinsulin molecules. The reaction is carried out at 8 ^{o}C for 12 h and reaches a yield of 85%. After completion of the refolding step, the refolding reagents are replaced with WFI and the protein solution is concentrated using a diafiltration unit (DF-103), which has a product recovery yield of 95% (5% of the protein denatures). The volume of the solution at this point is around 5000 L. Next, the human proinsulin is chromatographically purified in a hydrophobic interaction chromatography (HIC) column (C-102). The following operating assumptions were made: (1) the column is equilibrated for 30 minutes prior to loading, (2) the total resin binding capacity is 20 mg/ml, (3) the eluant volume is equal to 6 column volumes (CVs), (4) the total volume of the solutions for column wash, regeneration and storage is 15 CVs, (5) the protein of interest is recovered in 1 CV of eluant buffer with a recovery yield of 90%, and (6) the material of a batch is handled in three cycles.

Enzymatic conversion. The removal of the C-peptide from human proinsulin is carried out enzymatically (using trypsin and carboxypeptidase B) in a reaction tank (V-108). Trypsin cleaves at the carboxy terminal of internal lysine and arginine residues, and carboxypeptidase B removes terminal amino acids. The amount of trypsin used is rate-limiting and allows intact human insulin to be formed. Carboxipeptidase is added to a final concentration of 4 mg/L, while trypsin is added to a final concentration of 1 mg/L. The reaction takes place at 30 ^{o}C for 4 h and

reaches a conversion yield of 95%. The volume of the solution at this point is around 4300 L. Figure 3 displays schematically the various transformation steps that are required to convert fusion protein into active insulin (Vadim et al, 2004).

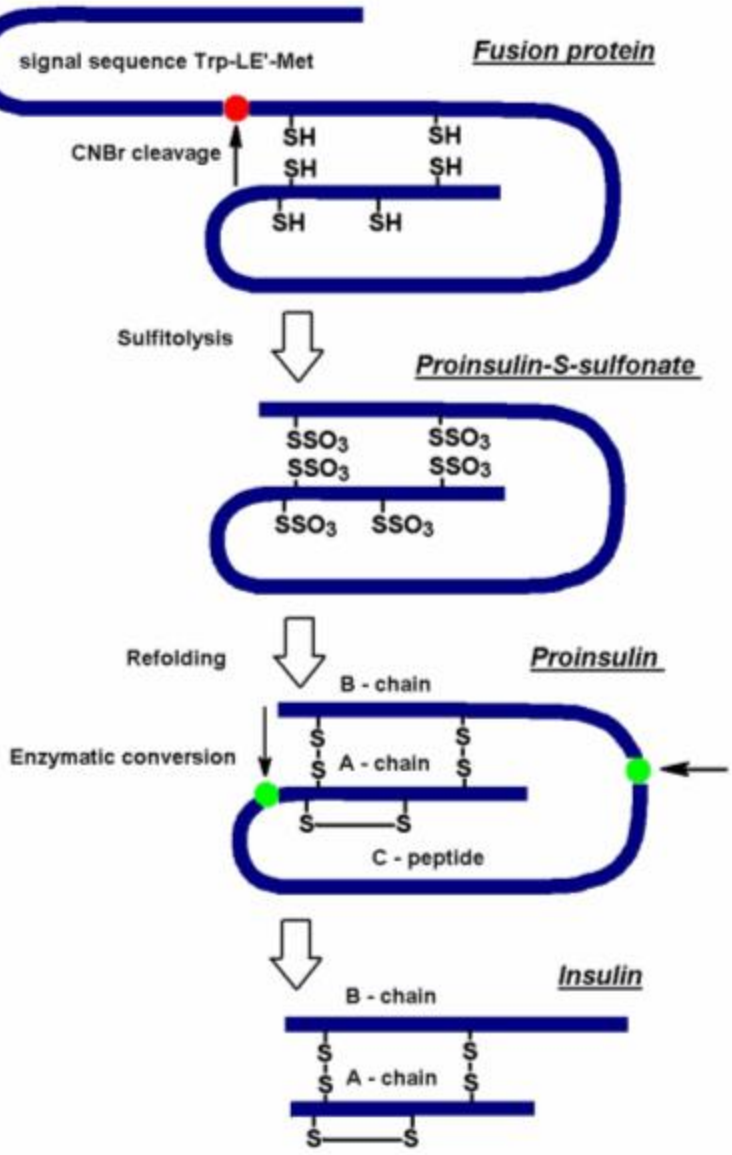

Figure 3 Transformation of fusion protein into insulin.

Final Purification Section A purification sequence based on multimodal chromatography, which exploits differences in molecular charge, size, and hydrophobicity, is used to isolate biosynthetic human insulin. A description of all the purification steps follows.

The enzymatic conversion solution is exchanged with WFI and concentrated by a factor of 4 in a diafilter (DF-103). An ion exchange column (C-103) is used to purify the insulin solution.

-

The following operating assumptions were made: (1) the column is equilibrated for 30 minutes prior to loading, (2) the total resin binding capacity is 20 mg/ml, (3) the eluant volume is equal to 8 CVs and the eluant is a 11.5 % w/w solution of NaCl in WFI, (4) the total volume of the solutions for column wash, regeneration and storage is 14 CVs, (5) the protein of interest is recovered in 1.5 CV of eluant buffer with a recovery yield of 95%, and (6) the material from each batch is handled in four cycles. The liquid volume at this point is around 1100 L.

Next, the ion exchange eluant solution is exchanged with WFI in a diafilter (DF-105) and is concentrated by a factor of 2.0. A recovery yield of 98% was assumed for this step (2% denatures).

The purification of the insulin solution proceeds with a reversed-phase high performance liquid chromatography (RP-HPLC) step (C-104). Detailed information on the use of RP-HPLC for insulin purification is available in the literature. Analytical studies with a variety of reversed-phase systems have shown that an acidic mobile phase can provide excellent resolution of insulin from structurally similar insulin-like components. Minor modifications in the insulin molecule resulting in monodesamido formation at the 21st amino acid of the A chain, or derivatization of amines via carbamoylation or formylation, result in insulin derivatives having significantly increased retention. Derivatives of this nature are typical of the kind of insulin-like components that are found in the charge stream going into the reversed-phase purification. The use of an acidic mobile phase results in elution of all the derivatives after the insulin peak, while the use of mildly alkaline pH results in derivatives eluted on either side of the parent insulin peak. An ideal pH for insulin purification is in the region of 3.0-4.0, since this pH range is far enough below the isoelectric pH of 5.4 to provide for good insulin solubility. An eluant buffer with an acetic acid concentration of 0.25 M meets these operational criteria because it is compatible with the chromatography and provides good insulin solubility. A 90% insulin yield was assumed in the RP-HPLC step with the following operating conditions: (1) the column is equilibrated for 30 minutes prior to loading, (2) the total resin binding capacity is 15 mg/ml, (3) the column height is 25 cm, (4) the eluant volume is equal to 6 CV and its composition is 25% w/w acetonitrile, 1.5% w/w acetic acid 73.5% w/w WFI, (5) the total volume of the solutions for column wash, equilibration, regeneration and storage is 6 CVs, and (5) the protein of interest is recovered in 1 CV of eluant buffer with a recovery yield of 90%.

The RP-HPLC buffer is exchanged with WFI and concentrated by a factor of 2.0 in a diafilter (DF-105) that has a product recovery yield of 98% (2% denatures). Purification is completed by a gel filtration chromatography column (C-105). The following operating assumptions were made: (1) the column is equilibrated for 30 minutes prior to loading, (2) the sample volume is equal to 5% of the column volume, (3) the eluant volume is equal to 4 CVs, (4) the total volume of the solutions for column wash, depyrogenation, stripping, and storage is 6

CVs, and (5) the protein of interest is recovered in 0.5 CV of eluant buffer with a recovery yield of 90%. The mobile phase is a solution of acetic acid.

Next, the same diafilter (DF-105) is used to concentrate the purified insulin solution by a factor of ten. The liquid volume at this point is around 500 L, which contains approximately 12.8 kg of insulin. This material is pumped into a jacketed and agitated reaction tank (V-111). Ammonium acetate and zinc chloride are added to the protein solution until each reaches a final concentration of 0.02 M (Datar and Rosen, 1990). The pH is then adjusted to between 5.4 and 6.2. The crystallization is carried out at 5 °C for 12 h. Insulin crystallizes with zinc with the following stoichiometry: $insulin_6$-Zn_2. Step recovery on insulin is around 90%.

The crystals are recovered with a basket centrifuge (BCF-101) with a yield of 95%. Finally, the crystals are freeze-dried (FDR-101). The purity of the crystallized end product is between 99.5 and 99.9 % measured by analytical high pressure liquid chromatography (HPLC). Approximately 11.5 kg of product is recovered per batch. The overall recovery yield is around 32%.

Material Balances and Environmental Assessment

Table 1 displays the raw material requirements in kg per year, per batch, and per kg of main product (MP = purified insulin crystals). Note the huge amounts of WFI, water, NaOH (0.5 M), H3PO4 (20% w/w), urea, acetic acid, formic acid, guanidine hydrochloride, and acetonitrile required per kg of final product. All of these materials end up in waste streams. The total waste-to-product ratio is 600,000:1.

Table 1 Raw material requirements (1 batch = 11.5 kg MP)

Raw Material	kg/yr	kg/batch	kg/kg MP
Glucose	782,238	4,888.989	432.250
Salts	71,428	446.428	39.470
Air	3,647,361	22,796.004	2,015.463
Ammonia	75,686	473.035	41.822
Water	9,854,820	61,592.627	5,445.588
WFI	67,027,394	418,921.212	37,038.073
NaOH (0.5 M)	3,990,587	24,941.168	2,205.123
H_3PO_4 (20% w/w)	4,405,176	27,532.351	2,434.217
TRIS Base	43,200	270.000	23.872
EDTA	10,427	65.170	5.762
Triton-X-100	3,035	18.970	1.677
CNBr	15,268	95.428	8.437
Formic acid	1,751,525	10,947.034	967.860
Urea	3,062,668	19,141.678	1,692.373
MrEtOH	98,660	616.625	54.518
NH_4HCO_3	5,551	34.691	3.067
$Na_2O_6S_4$	24,159	150.994	13.350

-

Sodium sulfite	48,318	301.987	26.700
Guanidine HCl	805,593	5,034.956	445.155
Sodium Chloride	778,024	4,862.649	429.921
Sodium Hydroxid	137,677	860.481	76.078
Acetic-Acid	2,262,278	14,139.240	1,250.092
Enzymes	3	0.021	0.002
Acetonitrile	767,182	4,794.886	423.930
Ammonium Acetat	181	1.133	0.100
Zinc Chloride	320	2.000	0.177
TOTAL	**99,668,761**	**622,929.756**	**55,075.077**

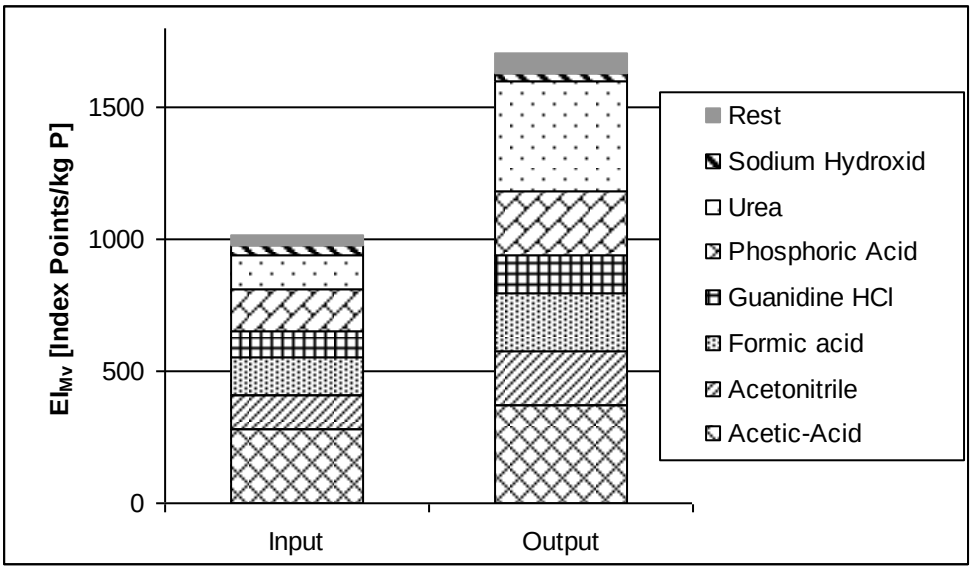

Figure 4 Environmental indices (EI_{Mv}) of the process.

Figure 4 shows the EI_{Mv} of the process. Acetic acid, phosphoric acid, formic acid, acetonitrile, urea and guanidine HCl dominate the input side. They are all based on oil and have some acute toxicity. Therefore, the impact groups Availability and Organisms contribute most to the overall environmental impact. The same substances also dominate the output EI. Here, there nitrogen and phosphorus content and their chemical oxygen demands is relevant and leads to the dominance of the impact group Water/Soil at the output side of the overall environmental impact.

In the base case, it was assumed that this waste is treated and disposed of. However, opportunities may exist for recycling some chemicals for in-process use and recovering others for off-site use. For instance, formic acid (HCOOH), acetonitrile, and urea are good candidates for recycling and recovery. Formic acid is used in large quantities (11 tons/batch) in the CNBr cleavage step (V-103) and it is removed using a rotary vacuum evaporator (CSP-101), along with small quantities of CNBr, H_2O, and urea. The recovered formic acid can be readily purified by distillation and recycled in the process. Around 2 tons per batch of urea is used for the dissolution of inclusion bodies (V-103) and 17 tons per batch is used in the first chromatography step (C-

-

101) to purify proinsulin$(S\text{-}SO_3)_6$ before its refolding. Approximately 90% of the urea appears in just two waste streams (Liquid Waste 4 & 7). It is unlikely that these urea-containing streams can be purified economically for in-process recycling. However, these solutions can be concentrated, neutralized, and shipped off site for further processing and utilization as a nitrogen fertilizer.

Approximately 4.8 tons per batch of acetonitrile is used in the reversed-phase HPLC column (C-104), and most of it ends up in the waste stream of the column (Liq. Waste 13) along with 6.8 tons of water, 1.85 tons of acetic acid, and small amounts of NaCl and other impurities. It is unlikely that acetonitrile can be recovered economically to meet the high purity specifications for a step so close to the end of the purification train. However, there may be a market for off-site use.

Scheduling and Resource Tracking

Figure 5 displays the equipment occupancy chart for three consecutive batches. The process batch time is approximately 11 days. This is the time required to go from the preparation of raw materials to final product for a single batch. However, since most of the equipment items are utilized for much shorter periods within a batch, a new batch is initiated every 2 days. Multiple bars on the same line within a batch (e.g., for DS-101, V-103, DF-101, DF-103, and DF-105) represent reuse (sharing) of equipment by multiple procedures. White space represents idle time. The equipment with the least idle time between consecutive batches is the *time (or scheduling) bottleneck* (DF-101 in this case) that determines the maximum number of batches per year. Its cycle time (approximately 41.5 h) is the minimum possible time between consecutive batches (also known as minimum process cycle time). This plant operates around the clock and processes 160 batches per year. The top six lines of Figure 5 correspond to cleaning-in-place (CIP) skids utilized to thoroughly clean the equipment. CIP skids are common bottlenecks in biopharmaceutical manufacturing facilities.

The execution in time of the various procedures and their operations can be visualized in detail through the operations Gantt chart (see Figure 6). Note, for instance, the operations of procedure P-8 (IB solubilization). The TRANSFER_IN operation in that procedure runs in parallel with the CENTRIFUGE_1 operation of the previous procedure (P-14). This is the case because while the IB slurry is being centrifuged as part of P-14, the concentrate (solids stream of the centrifuge) is being pumped into the vessel (V-103) of P-8. If this detail is not captured in the model, the identification of the scheduling bottleneck may be incorrect.

-

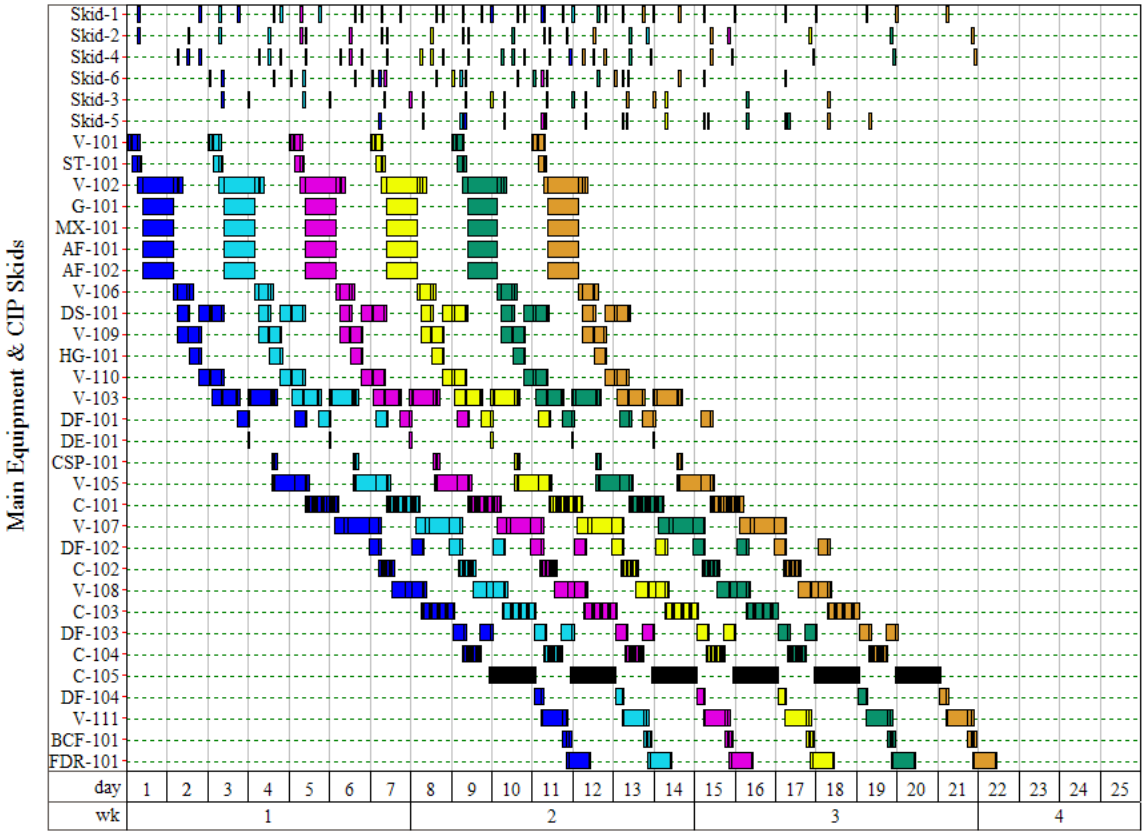

Figure 5 Equipment occupancy as a function of time for six consecutive batches.

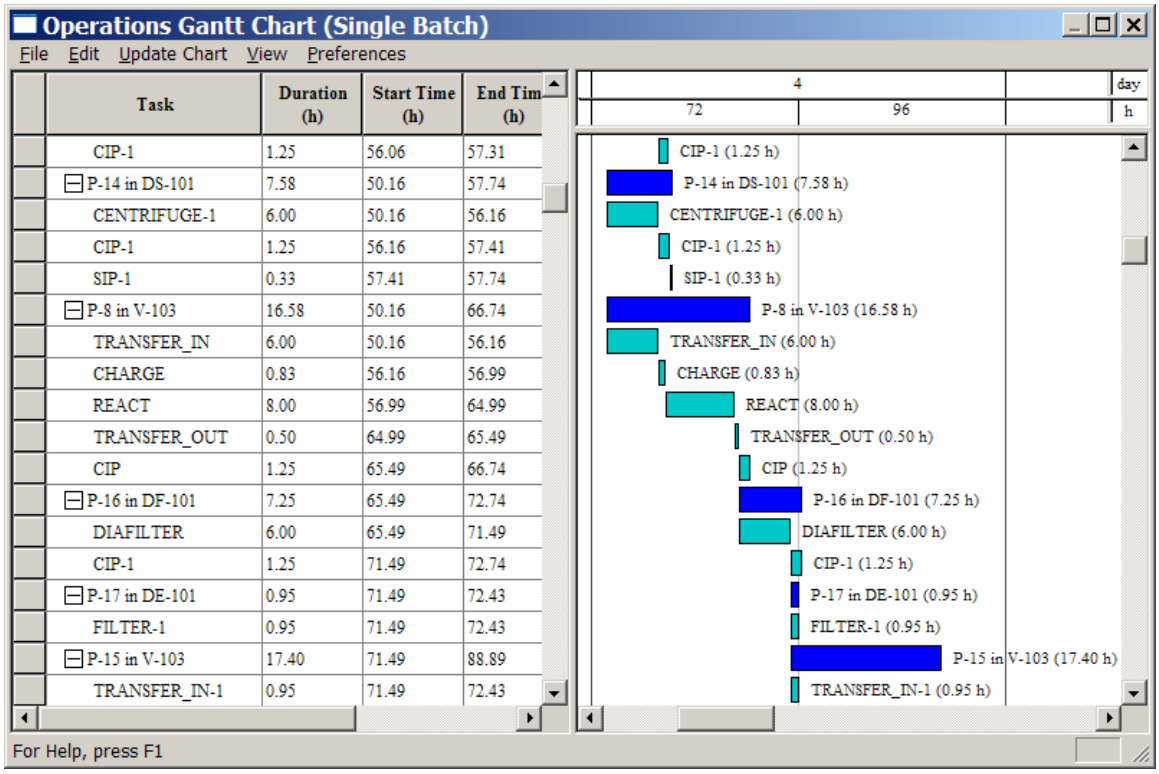

Figure 6 Operations Gantt chart.

Another characteristic of batch processing is the variable demand for resources (e.g., labor, utilities, and raw materials) as a function of time. For instance, Figure 7 displays the labor demand (expressed in number of operators) for 12 consecutive batches. Note that for short periods there is a need for up to 19 operators to be present. If that is not possible, certain operations will need to be delayed in order to distribute the demand for operators more evenly. In such a case, the limited resource becomes the time bottleneck. Demand for steam and other utilities may also become a time bottleneck. The results of Figure 7 are also useful in staffing a facility. If the facility is dedicated to manufacturing of a single product, then, the number of operators in each shift should be based on the peak demand during that shift. In multiproduct facilities, each production suite may employ a dedicated number of operators and utilize floating operators during periods of peak demand.

-

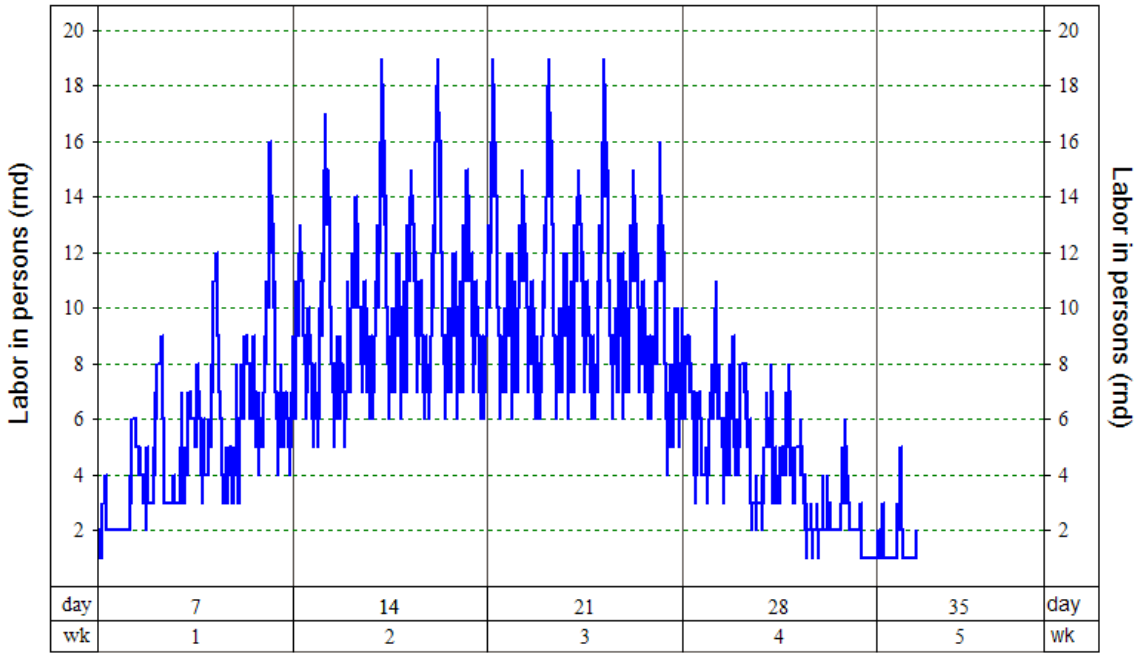

Figure 7 Labor demand as a function of time for ten consecutive batches.

Economic Evaluation

Table 2 shows the key economic evaluation results generated by using the built-in cost functions of SuperPro Designer. For a plant of this capacity, the total capital investment is $145 million. The unit production cost is $67.2/g of purified insulin crystals. Assuming a selling price of $120/g, the project yields an after-tax internal rate of return (IRR) of 63.5% and a net present value (NPV) of $397 million (assuming a discount interest of 7%). In the US, the retail price of vials that contain 40 mg of insulin is around $25, which is equivalent to $625/g of active insulin. Therefore, a selling price of $120/g of bulk insulin corresponds to around 20% of the retail selling price of the final product, which is reasonable considering the additional cost and profit margins for formulation, packaging, distribution, etc. Based on these results, this project represents a very attractive investment. However, if amortization of up-front R&D costs is considered in the economic evaluation, the numbers change drastically. For instance, a modest amount of $150 million for up-front R&D cost amortized over a period of 10 years reduces the IRR to 21%.

Figure 8 breaks down the operating cost. The cost of consumables is the most important, accounting for 38.1% of the overall manufacturing cost. This represents the expense for periodically replacing the resins of the chromatography columns and the membranes of the membrane filters. The cost of raw materials lies in the second position accounting for 29.1% of the overall cost. The facility overhead accounts for 17.7% of the total cost. This mainly represents the depreciation and maintenance of the facility. Treatment and disposal of waste materials account for 7.2% of the total cost. As mentioned in the material balance section, recycling and reuse of some of the waste materials may reduce this cost. Labor lies in the fifth position

-

accounting for 6.2% of the total cost. Approximately 60 operators are required to run the plant around the clock, supported by 12 scientists for QC/QA work. The cost of utilities is only 0.2% because it comprises only electricity and the small amounts of heating and cooling required. The cost of purified water is treated as a raw material and not as a utility.

Table 2 Key economic evaluation results.

Direct Fixed Capital	$117.1 million
Total Capital Investment	$145.2 million
Plant Throughput	1,810 kg/year
Manufacturing Cost	$121.7 million/year
Unit Production Cost	$67.2/g
Selling Price	$120/g
Revenues	$217.1 million/year
Gross Profit	$95.4 million/year
Taxes (40%)	$38.2 million/year
Net Profit	$68.4 million/year
IRR (after taxes)	63.5%
NPV (for 7% discount interest)	$397 million

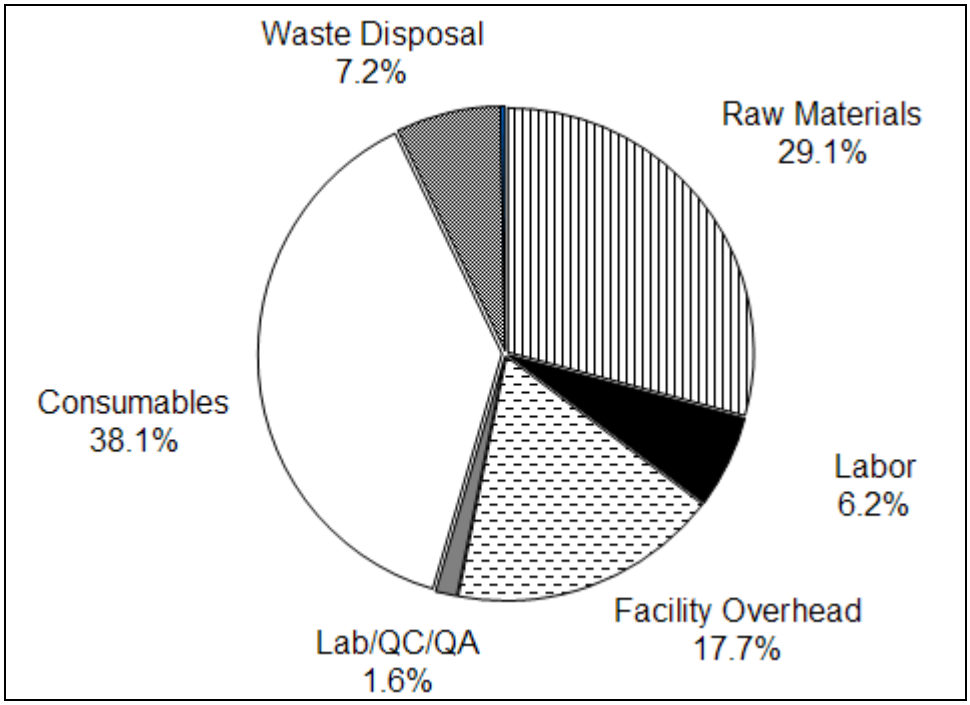

Figure 8 Breakdown of manufacturing cost.

Figure 9 displays the cost distribution per flowsheet section. Only 5.8% of the overall cost is associated with fermentation. The other 94.2% is associated with the recovery and purification sections. This is common for high value biopharmaceuticals that are produced from recombinant *E. coli*. Most of the cost is associated with the reactions section because of the large amounts of expensive raw materials and consumables that are utilized in that section.

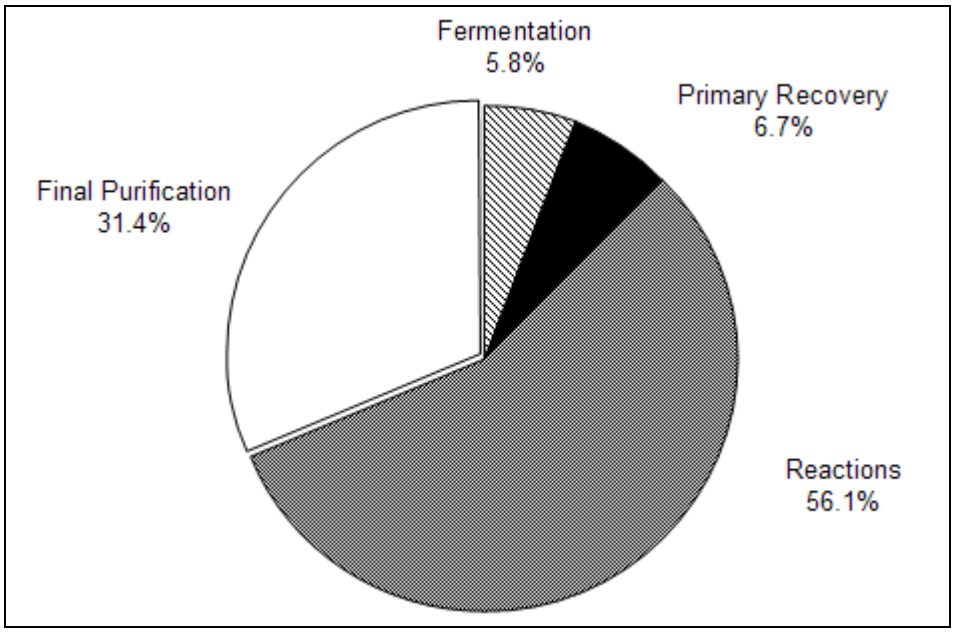

Figure 9 Cost distribution per flowsheet section.

Table 3 for each consumable displays its annual amount, unit cost, annual cost, and contribution to the overall consumables cost. The Gel Filtration resin is the most expensive consumable, followed by the first S-Sepharose resin and the HIC resin. Gel filtration accounts for 10% of the overall manufacturing cost. Replacement of the gel filtration step with an alternative and more efficient chromatography step can have a big impact on the manufacturing cost and should be considered in future versions of this process.

Table 3 Cost of Consumables

Consumable	Annual Amount	Unit of Measure (UOM)	Unit Cost $/UOM	Annual Cost ($)	%
UF Membrane	4,786	m2	800	3,829,000	8.46
HIC Resin	4,312	L	2,000	8,624,000	19.05
Gel Filtration Resin	16,236	L	800	12,989,000	28.69
DEF Cartridge	3,840	item	800	3,072,000	6.78
S-Seph-1 Resin	8,285	L	1,200	9,942,000	21.96
S-Seph-2 Resin	2,222	L	1,500	3,333,000	7.36
RP-HPLC-Resin	1,745	L	2,000	3,490,000	7.71

-

TOTAL				45,278,000	100.00

Finally, table 4 for each raw material displays its price, annual amount, annual cost and contribution to the overall raw materials cost. WFI, acetic acid, urea, and H_3PO_4 (20% w/w) are the major contributors to the raw materials cost. The solution of H_3PO_4 is used for equipment cleaning.

Table 4 Cost of Raw Materials

Raw Material	Unit Cost ($/kg)	Annual Amount (kg)	Annual Cost ($)	%
Glucose	0.600	782,238	469,000	1.32
Salts	1.000	71,428	71,000	0.20
Ammonia	0.700	75,686	53,000	0.15
Water	0.050	9,854,820	493,000	1.39
WFI	0.100	67,027,394	6,703,000	18.92
NaOH (0.5 M)	0.500	3,990,587	1,995,000	5.63
H_3PO_4 (20% w/w)	1.000	4,405,176	4,405,000	12.43
TRIS Base	6.000	43,200	259,000	0.73
EDTA	18.500	10,427	193,000	0.54
Triton-X-100	1.500	3,035	5,000	0.01
CNBr	11.000	15,268	168,000	0.47
Formic acid	1.600	1,751,525	2,802,000	7.91
Urea	1.520	3,062,668	4,655,000	13.14
MrEtOH	3.000	98,660	296,000	0.84
NH_4HCO_3	1.000	5,551	6,000	0.02
$Na_2O_6S_4$	0.600	24,159	14,000	0.04
Sodium sulfite	0.400	48,318	19,000	0.05
Guanidine HCl	2.150	805,593	1,732,000	4.89
Sodium Chloride	1.230	778,024	957,000	2.70
Sodium Hydroxid	3.500	137,677	482,000	1.36
Acetic-Acid	2.500	2,262,278	5,656,000	15.96
Enzymes	500,000.000	3	1,691,000	4.77
Acetonitrile	3.000	767,182	2,302,000	6.50
Ammonium Acetat	15.000	181	3,000	0.01
Zinc Chloride	12.000	320	4,000	0.01
TOTAL		**99,668,761**	**35,433,000**	**100.00**

Throughput Increase Options

In the base case, a new batch is initiated every 48 h. Most of the equipment items, however, are utilized for less than 24 h per batch (see Figure 5). If the market demand for insulin grows, this provides the opportunity for increasing plant throughput without major capital expenditures. A

-

realistic improvement is to initiate a batch every 24 h. This will require a new fermentor of the same size whose operation will be staggered relative to the existing unit so that one fermentor is ready for harvesting every day. Such a production change will also require additional equipment of the following types: (1) disk-stack centrifuges to reduce the occupancy of DS-101 to less than 24 h; (2) two new reactors to reduce the occupancy of V-103 and V-107; a new gel filtration chromatography column, and (3) membrane filters to reduce the occupancy of DF-101, DF-102, and DF-103.

The additional capital investment for such a retrofit is around $30-$40 million. This additional investment will allow the plant's capacity to be doubled, and the new unit production cost will be around $62/g. The reduction in the unit production cost is rather small because the majority of the manufacturing cost is associated with raw materials, consumables, and waste disposal that scale approximately linearly with production.

Conclusions

In this chapter, we have analyzed the production of BHI from recombinant E. coli. The development of the process was based on information available in the literature. The work was facilitated using SuperPro Designer, a comprehensive process simulator. The analysis has clearly shown that most of the cost for manufacturing high-value biopharmaceuticals with recombinant E. coli is associated with the recovery and purification of the product. The large number of conversion and separation steps required to recover and purify the product lead to a low recovery yield of 32% and a huge waste-to-product ratio (600,000:1). Improved processes that result in reduced manufacturing cost can greatly contribute towards the effort of making insulin accessible to diabetics in the developing nations.

REFERENCES

Dalton, L. W. (2004). Drugs for Diabetes, *Chemical & Engineering News*, October 25, 59-67.

Barfoed, H.C. (1987). Insulin Production Technology, *Chem. Eng. Prog*, 83, 49-54.

Bjurstrom Ed (1985). Biotechnology, *Chemical Engineering*, February issue.

Chan. M.Y.Y., M. Hoare, and P. Dunnill (1986). The Kinetics of Protein Precipitation by Different Reagents, *Biotechnology and Bioengineering*, Vol. XXVIII, 387-393.

Crueger W. and A. Crueger (1989). *BIOTECHNOLOGY – A Textbook of Industrial Microbiology*, 2nd edition, Sinauer Associates, Inc., Sunderland, MA.

Data, R. and C.-G. Rosen (1990). Downstream process economics, 741-793. In *Separation Processes in Biotechnology*, (Asenjo, J.A, ed.), Marcel Dekker, NY.

-

DeYoung, H.G. (1996). Multiple Choices for Monoclonal Production. *Genetic Engineering News*, *16 No. 14.* 12-33.

Dlouhy, P.E. and d.A. Dahlstrom (1968). Continuous Filtration in Pharmaceutical Processing, *Chemical Engineering Progress*, 64, No. 4, 116-121.

Fish, W.W., A. Danielson, K. Nordling, S.H. Miller, C.F. Lam, and I. Bjork (1985). Denaturation Behavior of Antithrombin in Guanidinium Chloride, *Biochemistry*, 24, 1510-1517.

Garrett, D.E. (1989). *Chemical Engineering Economics*, Van Nostrand Reinhold, New York.

Kehoe, J.A. (1989). The Story of Biosynthetic Human Insulin, 45-49. In *Frontiers in Bioprocessing*, (Sikdar, S.K, M. Bier, and P. Todd, editors.), CRC Press, Inc., Boca Raton, FL.

Leser, E.W. and J.A. Asenjo (1992). Rational design of purification processes for recombinant proteins, *Journal of Chromatography*, 584, 43-57.

Ladisch, M.R. and Kohlmann, K.L. (1992). Recombinant Human Insulin, *Biotechnology. Progress*, 8, 469-478.

Palmer, D.E. (1977). High Purity Protein Recovery, *Process Biochemistry*, June, 24-28.

Peters, Max S., and Klaus D. Timmerhaus (1991). *Plant Design and Economics for Chemical Engineers*, 4th edition, McGraw-Hill, NY.

Petrides, D.P., C.L. Cooney, and L.B. Evans (1989). An Introduction to Biochemical Process Design, in *Chemical Engineering Problems in Biotechnology*, (Shuler, M.L, ed.), Vol. 1, AIChE, 351-372.

Petrides, D.P. (1994). BioPro Designer – An Advanced Computing Environment for Modeling and Design of Integrated Biochemical Processes, *Computers and Chemical Engineering*, 18 (SS): S621-S625.

Petrides, D.P., E. Sapidou, and J. Calandranis (1995). Computer-Aided Process Analysis and Economic Evaluation for Biosynthetic Human Insulin Production – A Case Study, *Biotechnology and Bioengineering*, 5, 529-541.

Reisman, H.B. (1988). *Economic Analysis of Fermentation Processes*. CRC Press, Inc., Boca Raton, Fla.

Roberts L.R. (1979). In *Encyclopedia of Chemical Processing and Design*, 8, Marcel Dekker, New York, 324-333.

Taylor, G., M. Hoare, D.R. Gray, and F.A.O. Marston (1986). Size and Density of Protein Inclusion Bodies, *Bio/Technology*, 4, June.

-