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DESING OF A TWO STAGE REACTOR SYSTEM FOR PROTEIN PRODUCTION USING PLASMID ENCODED E. COLI AND WHEY PERMEATE AS A COMBINATION SUBSTRATE AND INDUCER

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ABSTRACT

Whey disposal problems in the United States have increased over the past twenty years. Few economically attractive options are available for whey utilization.

The Cornell Excretion System (CES) uses a genetically engineered cell to produce plasmid-encoded proteins in a two stage continuous flow reactor system. The host has been E. Coli RB791 with a pKN plasmid. The target protein gene is under control of the tac promoter, which is induced by lactose. Experimental systems have demonstrated the feasibility of continuous production of the β-lactamase enzyme, at high levels (>0.5 g/l), with high levels of excretion (>90%), and high purity (>50%). Induction of the tac promoter leads to increasing plasmid copy number and to eventual cell death. Mutants not responding to induction have a selective advantage. However, lactose (because it is both inducer and carbon/energy source) would prevent the formation of such mutant.

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*. Current afiliation: Baxter Biotech -Fenwal Division. Parque ITABO. Haina, República Dominicana. 809-542-2752, 809-542-2334 (FAX).

produces commercial enzymes using the CES, and lactose from whey permeate was designed at six different scales (0.013 million pounds of milk permeate per day, and 0.50-1.03 million pounds of whey permeate per day). Based on molar production rates (0.5 fraction of molar rate), yield factors (150 units of β-Lactamase/mg cell, 0.35 g cells/g lactose), and other assumptions, profitability and sensitivity analysis were performed. Under the hypothesis that the plant would produce Bacillus protease, glucose isomerase, and calf rennet for 65, 30, and 5% of the total operating time, rates of return of 0 (milk permeate scale) to 26-35% (whey permeate scale), and total capital investments (TCI) of 6.4 (milk permeate scale) to 56.6-90.9 (whey permeate scale) million dollars were obtained. When it was assumed that the milk permeate scale was producing calf rennet for 100% of its total operating time, a rate of return of 73%, and a total capital investment of 6.4 million dollars were calculated. It was concluded that there are economic incentives for the determination of the behavior of the CES when using lactose as an inducer to produce such enzymes.

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Enzymes have much higher economic value than products from previously proposed whey utilization schemes. A plant that produces commercial enzymes using the CES, and lactose from whey permeate was designed at six different scales (0.013 million pounds of milk permeate per day, and 0.50-1.03 million pounds of whey permeate per day). Based on molar production rates (0.5 fraction of molar rate), yield factors (150 units of β-Lactamase/mg cell, 0.35 g cells/g lactose), and other assumptions, profitability and sensitivity analysis were performed. Under the hypothesis that the plant would produce Bacillus protease, glucose isomerase, and calf rennet for 65, 30, and 5% of the total operating time, rates of return of 0 (milk permeate scale) to 26-35% (whey permeate scale), and total capital investments (TCI) of 6.4 (milk permeate scale) to 56.6-90.9 (whey permeate scale) million dollars were obtained. When it was assumed that the milk permeate scale was producing calf rennet for 100% of its total operating time, a rate of return of 73%, and a total capital investment of 6.4 million dollars were calculated. It was concluded that there are economic incentives for the determination of the behavior of the CES when using lactose as an inducer to produce such enzymes.

1. Introduction, Scope and Methodology.

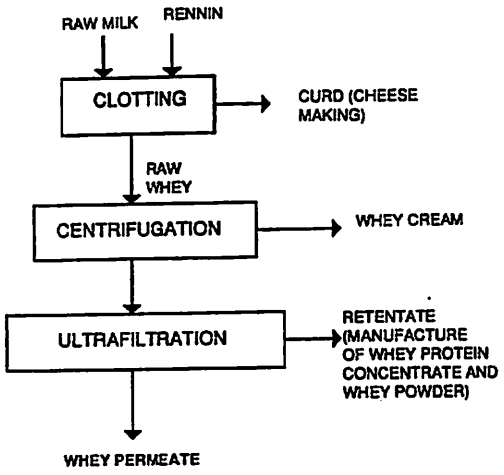
Milk and whey permeate are 5% lactose solutions mostly discarded from cheese manufacturing plants (figure 1.1). Their pH and mineral concentration depend on the type of cheese that is

produced. Over the last twenty years whey production in the United States has increased principally because of two trends:

- Increase in cheese consumption[1]. In 1989, total cheese shipments were 13.5% bigger over the previous year. Such increase is associated with higher pizza consumption which uses mozzarella cheese as principal ingredient[2].

- Decrease in milk consumptionn[3]. (1)..

Whey Permeate[8]:



Milk Permeate[9]:

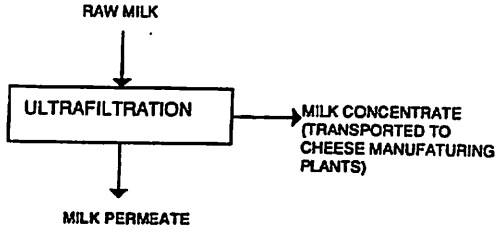


Figure 1.1 Permeate Sources Flowsheet

In the US, roughly 45% of the whey produced is wasted[4]. The preferred disposal methods are open land discharge and sewage dumping. The first is limited by land availability within 20 miles of the cheese plant, which is regarded as the maximum hauling distance for keeping costs under control[5]. Any affluent with Biological Oxygen Demand (BOD) greater than 200 requires a surcharge in municipal sewage plants. Whey has a BOD greater than 30,000[6]. The problems with the sewage option are increasing surcharge (in some cases they have doubled or quadrupled), and the failing of the treatment plants (most of them malfunction at least once a year and for 25% of the time are less than 75% efficient[7].

The Cornell Excretion System is considered a feasible way of producing proteins. It consists of E. coli (RB791(pKN)) with the tac (hybrid trp-lac) promoter, which is induced by lactose analogs such as isopropyl β-D-thiogalactoside (IPTG). After induction, E. coli can not normally synthesize outer membrane proteins and becomes leaky. That leads to high levels of protein excretion (table 1.1). The leaky phenotype results from increasing plasmid copy number and leads to eventual cell death. These mutants growth normally and produce no product[8,9,10]. Although the two stage system circumvent this problem, the potential use of lactose is attractive since it would act as both inducer and carbon/energy source, which would further suppress the formation of mutants.

Table 1.1 The Cornell Excretion System

Basis: Induction of selected strains reduces the rate of outer membrane proteins synthesis, which leads to enzyme excretion.

Characteristics:

Extracellular Protein Concentration:	> 0.5 g/lit
Purity:	50%
Operation:	> 50 days
Expression Level:	25% of cellular protein
Excretion:	90% of ß-Lactamase
Reactors:	Two stage chemostat
Mutants:	< 0.1% in second fermentor
Strain:	RB791(pKN)
Inducer:	IPTG
Promoter:	tac
Medium:	glucose (2-4 g/lit) in Tanaka Me.
Cell Concentration:	< 4 g/lit

Experimental data of the CES is only available for IPTG as inducer under certain conditions. Given the lactose over production mentioned above, a plant that utilize it as inducer in the CES for the manufacturing of high volume proteins is very reasonable. It would convert part of the lactose into glucose and galactose to use them for cell growth. A simplified flowsheet of such plant is presented on figure 1.2.

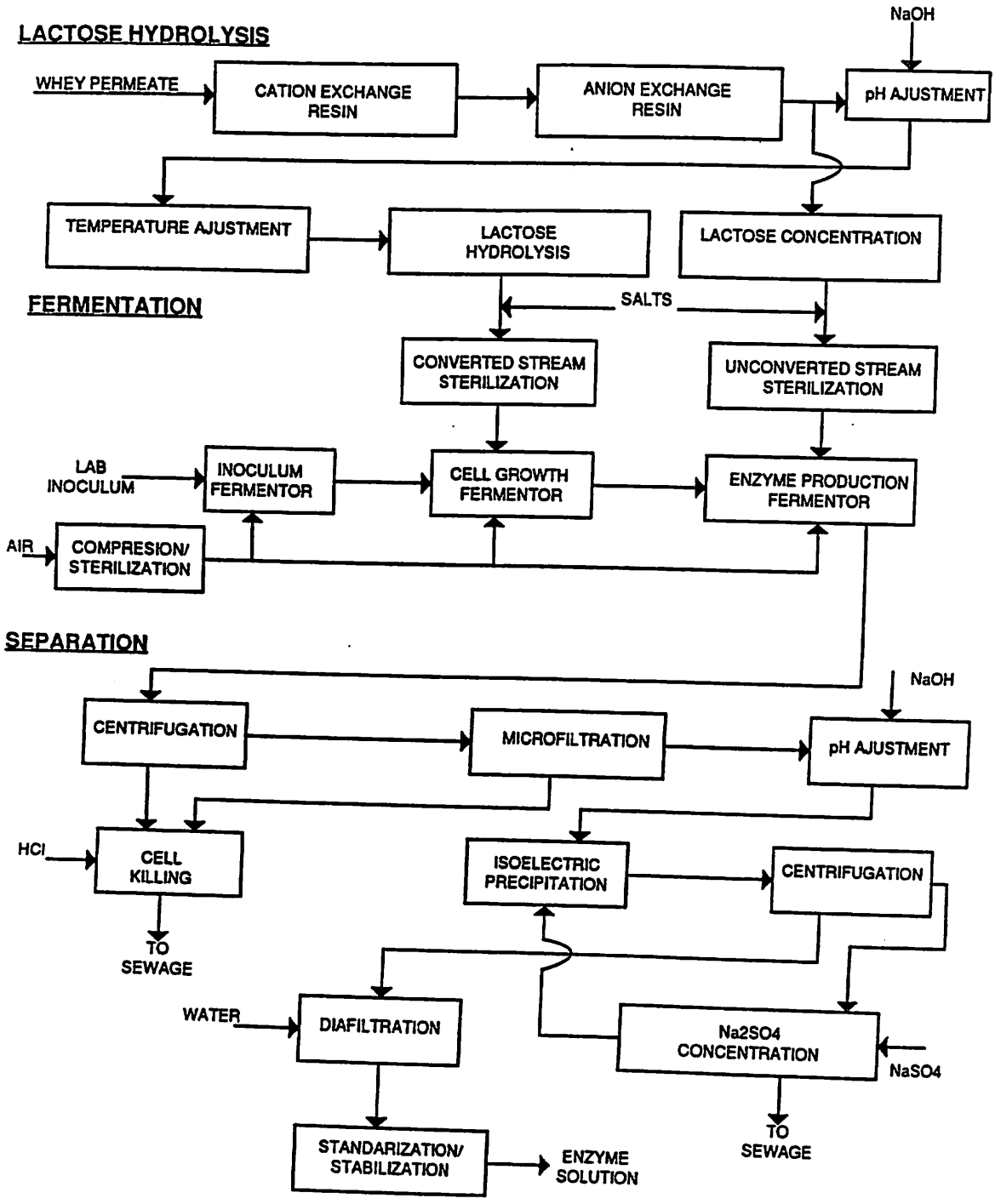


Figure 1.2 Enzymes' Production (Simplified Flowsheet)

The scope of this project was to:

- Design a plant that would produce commercial enzymes using the CES and whey permeate as feed.

- Find out the profitability and perform sensitivity analysis on such plant.

- Determine if this is a feasible alternative for dealing with the whey disposal problems in the United States.

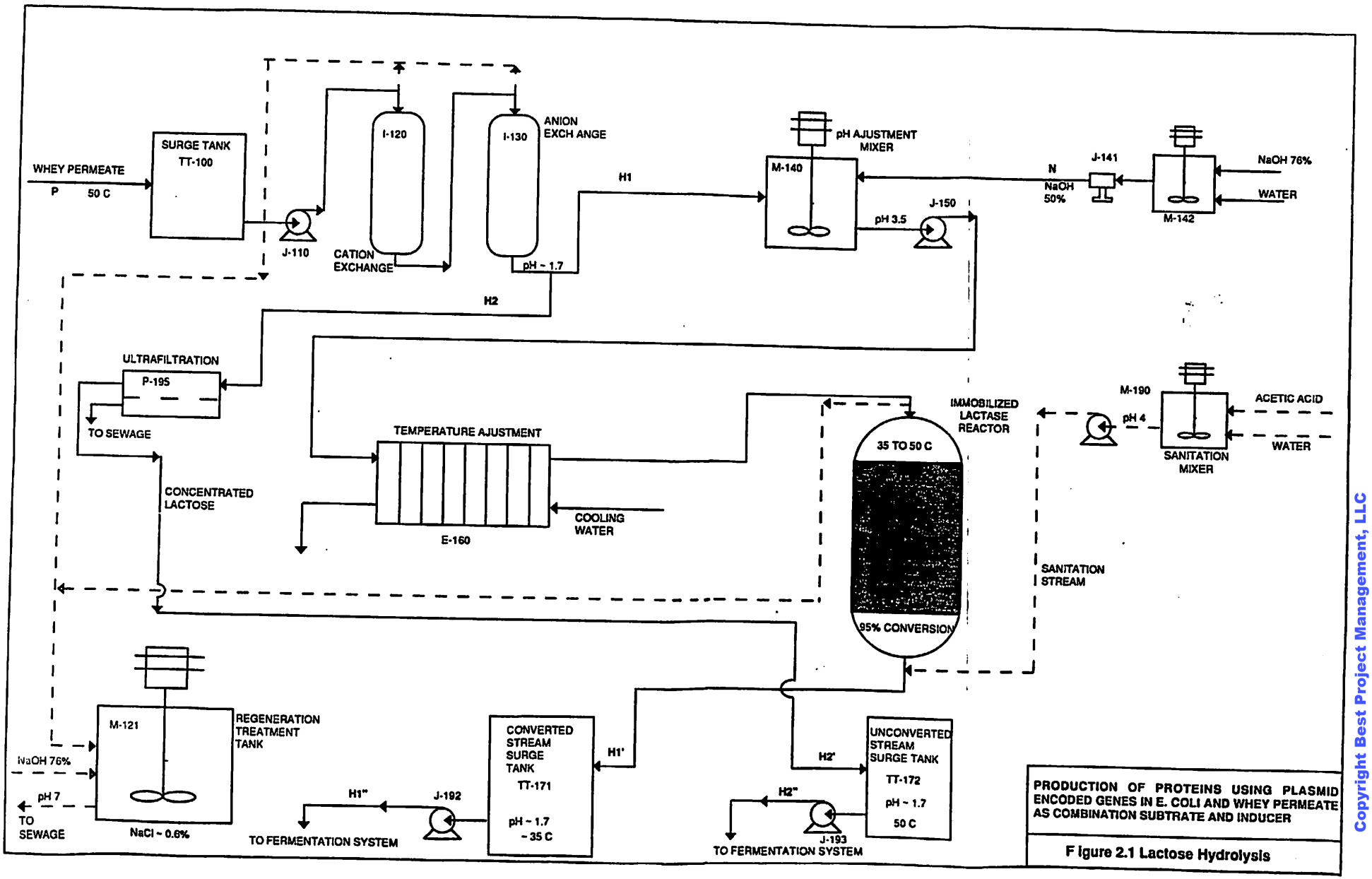
2. Lactose Hydrolysis.

The Corning Hydrolysis system was selected for the lactose hydrolysis. It was the first commercial process for such purpose available in the United States[11]. Its good performance has been demonstrated at pilot[12,13] and at industrial scale[14,15]. It consists of an immobilized lactase reactor operating from 35 to 50 °C to maintain constant conversion. To increase its operating life, the whey is ultrafiltrated and demineralized. Since the feed of the plant is permeate, the UF step is unnecessary. The proposed flowsheet is shown on figure 2.1. The streams are detailed on table 2.1.

Table 2.1 Streams' Flows and Compositions (Lactose Hydrolysis System) (Scale: 0.5 Million Pounds Whey Permeate/Day)

Stream	pH	Temp. (°C)	Glucose (g/L)	Galactose (g/L)	Lactose (g/L)	NaOH (% -w/w-)	Flow (kg/hr)
P	~6	50	0	0	47	0	10732
H1	1.7	50	0	0	47	0	5366
H1'	3.5	35-50	23.48	23.48	2.35	-	5374
H1"	3.5	35-50	23.48	23.48	2.35	-	4729*
H2	1.7	50	0	0	47	0	5366
H2'	1.7	50	0	0	120	0	2100
H2"	1.7	50	0	0	120	0	1848*
N	-	20	0	0	0	50	8

* The lactose hydrolysis system would work 21 hr/day and the fermentation system 24 hr/day.
- Not calculated.



Demineralization (by ion exchange) is necessary to increase the operating life of the immobilized lactase. Once demineralized, the permeate is split and part of it (H1) is converted into glucose and galactose. The hydrolysis is performed by lactase immobilized on porous glass. Characteristic of the reactor are presented on table 2.2.

Table 2.2 Immobilized Lactase Reactor Characteristics

Rate expression:	V=kES / (S+Km(1+P/Ki))
Sanitation:	Acetic acid solution (pH 4)
Enzyme carrier:	Porous glass
Average pore diameter:	370 A
Pellet diameter:	0.46 mm
Void fraction:	0.35 (bench scale)
Carrier total area:	~ 82 m^2/g
Apparent activity:	300 lactase units/g
Optimum pH:	3.5
Operating temperatures:	35 to 50 °C
Deactivation energy:	12 kcal/mol
Half life (50 °C):	62 days
Enzyme operating life:	559 (predicted)
Enzyme operating life:	365 (experimental)
External mass transfer:	No observed
Internal mass transfer:	No observed
Axial dispersion:	Insignificant

The pH of the demineralized stream (H1) is approximately 1.7. It is raised to 3.5 (optimum pH of the reactor) with sodium hydroxide (figure 2.1). As regard to the temperature, the best operating strategy starts the reactor at 35 °C and increases the temperature to 50 °C to maintain constant conversion. Based on the half live at 50 °C, and the deactivation energy (table 2.1), the time for the 35-50 °C cycle was estimated as 559 days[16,17]. However, the times obtained in commercial operations were considerably lower (approximately one year)[18]. In spite of that, they are adequate for economic operation.

The kinetics of lactose hydrolysis is affected by galactose inhibition. The rate expression is presented on table 2.2. Values of the turnover number (k=6E-5 mol/units/hr), Michelis constant (Km=0.0528 mol/l), and inhibition constant (ki=0.0054 mol/l) are given in the references[19].

As regard to the sanitation of the immobilized lactase reactor, it is achieved by back flushing the reactor with an acid solution brought to pH 4 by the addition of acetic acid[20]. This sanitation and the resin regeneration are the reasons why the lactose hydrolysis part of the plant would only work 21 hours per day.

Lactose is concentrated to alter the nutrient ratio to both fermentors, and to lower the enzyme dilution in the second one. Since the stream has already been sterilized in the cheese plant, and passed though ion exchange beds, the UF equipment would perform under very favorable conditions. Besides, lactose is only

concentrated up to ~120 g/l. This avoids concentration polarization problems.

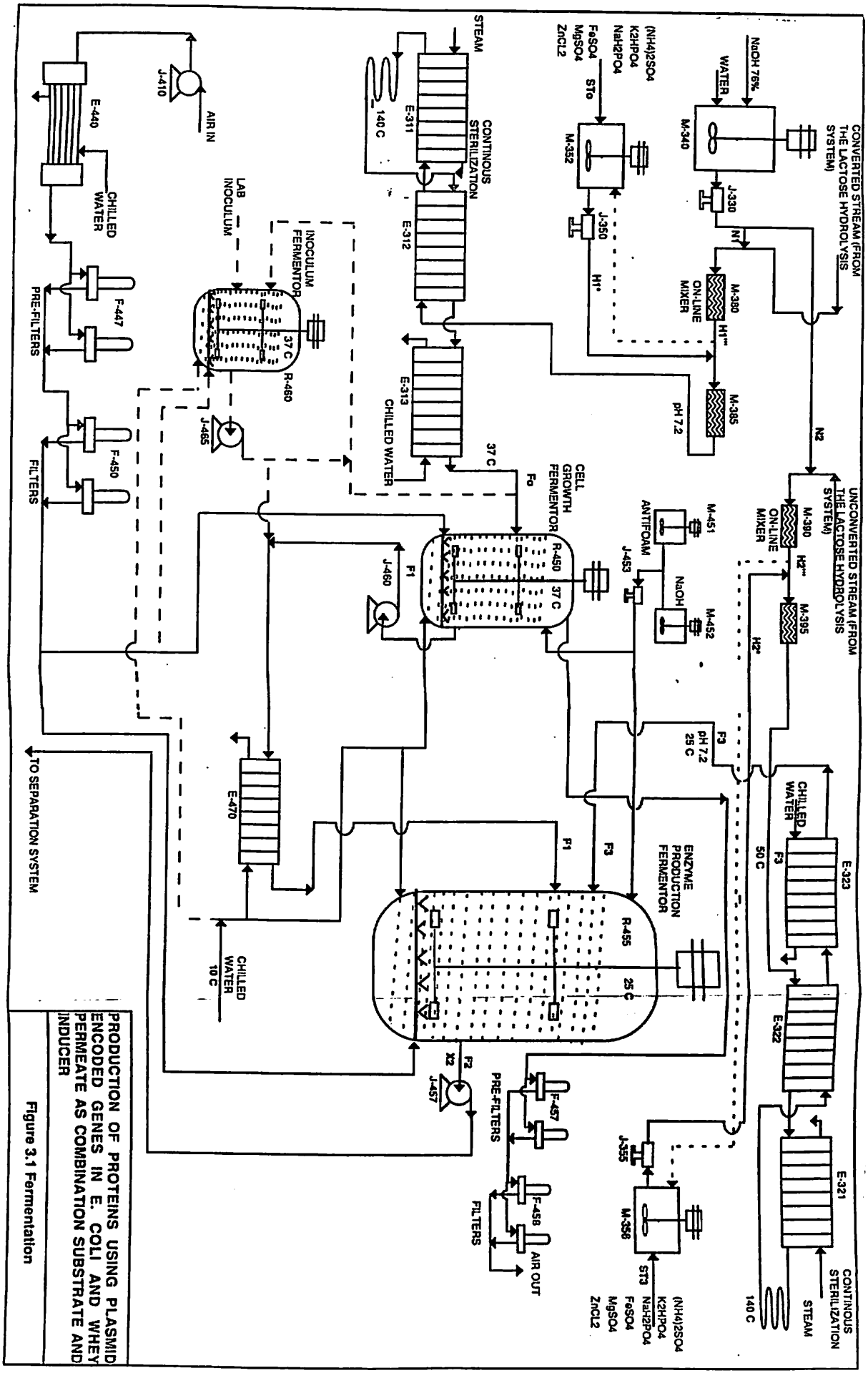
3. Fermentation.

3.1 Medium Formulation.

The pH of the converted stream (glucose + galactose) and unconverted streams (lactose) is adjusted to 7.2 with sodium hydroxide in on-line mixers (figure and table 3.1). Next, based on elements' material balances, salts necessary for metabolism and growth are added. It is known that some salts in the medium have limited solubility, or may form insoluble compounds ($Ca_2(PO_4)_3$). In defining the concentrations, precaution were taken to avoid precipitation. Calcium ions are maintained at very low levels because the permeate stream have already been demineralized. Besides, the constant streams' movement assures that precipitation problems will not be present.

Table 3.1 Streams' Flows and Compositions (Fermentation System) (Scale: 0.50 Million Pounds of Whey Permeate/Day)

Stream	Cell C. (g/L)	Glucose (g/L)	Galactose (g/L)	Lactose (g/L)	ß-Lactamase (g/L)	Flow (kg/hr)
Fo	0	23.28	23.48	2.35	0	4811
F1	18.69	0.0025	0.0025	2.35	0	4811
F2	25.31	~0	~0	0.057	4.74	6759
F3	0	0	0	120	0	1948
H1'''	0	23.48	23.48	2.35	0	4729
H1*	0	23.48	23.48	2.35	0	13
H2'''	0	0	0	120	0	1848
H2*	0	0	0	120	0	12



PRODUCTION OF PROTEINS USING PLASMID ENCODED GENES IN E. COLI AND WHEY PERMEATE AS COMBINATION SUBSTRATE AND INDUCER

Figure 3.1 Fermentation

3.2 Sterilization.

For the whey permeate stream there is the advantage of low microbial growth in the incoming stream because it has already being sterilized. In spite of this favorable situation, the sterilization system was designed assuming worst case scenarios. Plate heat exchangers were selected for the heat transfer operations.

3.3 Fermentation.

The unconverted stream is fed to the enzyme production fermentor and the lactose acts as inducer and as nutrient for cell growth and enzyme production. Once induced, the cells are directed to produce the desired enzymes. In doing so, they alter the synthesis of outer membrane proteins and become leaky. That leads to enzyme excretion. Since most of the cells die in this process, new ones have to be fed form the cell growth fermentor to sustain steady state operations[21].

It is assumed that small amounts of lactose (~2.35 g/l) fed to the cell growth fermentor will not induce the cells. This lactose concentration appears because the immobilized lactase reactor is operated at 95% conversion. Higher conversions increase disproportionately the size of such reactor.

The basis of the fermentation part of the plant is the Cornell Excretion System (CES) (table 1.1), which has been tested with glucose as cell growth nutrient. In the proposed plant, it is assumed the E. coli will use glucose and galactose for cell growth. Experimental basis for this assumption have appeared in

the literature[22]. In such reference it is mentioned that in batch fermentation, galactose was only used after all the glucose was depleted. However, in continuous operations, both carbohydrates were consumed at the same time. This was attributed to the small steady state glucose concentrations which were not sufficient to cause galactose inhibition[23,24].

Air sterilization would be performed by membrane filters. To avoid the releasing of genetically modified microorganisms to the environment, exhausted air is pre-filtered and filtered.

4. Separation.

The separation system is a compromise of different processes and limitations a that have been described for individuals enzymes[25,26,27,28]. It accounts for old and new trends in enzyme separation[29,30,31,32]. Some laboratory separations of the CES were performed using chromatography. Such operation was not included in the plant because its cost at industrial scale has been estimated at $5 per gram of product protein[33], and most of the produced enzymes have prices between 100 and 250 $/kg[34].

The cell are separated form the both (F2) by centrifugation. Cell concentrations of 14% (dw) are achieved in both centrifuges (F3 and C) (figure 4.1 and table 4.1). The centrifuged stream (F4) is sterilized in microfiltration membranes (figure 4.1). Once the stream is sterilized (FM1), the pH is adjusted to the isoelectric point of the enzyme that is being produced. Enzyme denaturalization is avoid by employing very dilute solutions (N3) of NaOH and HCl.

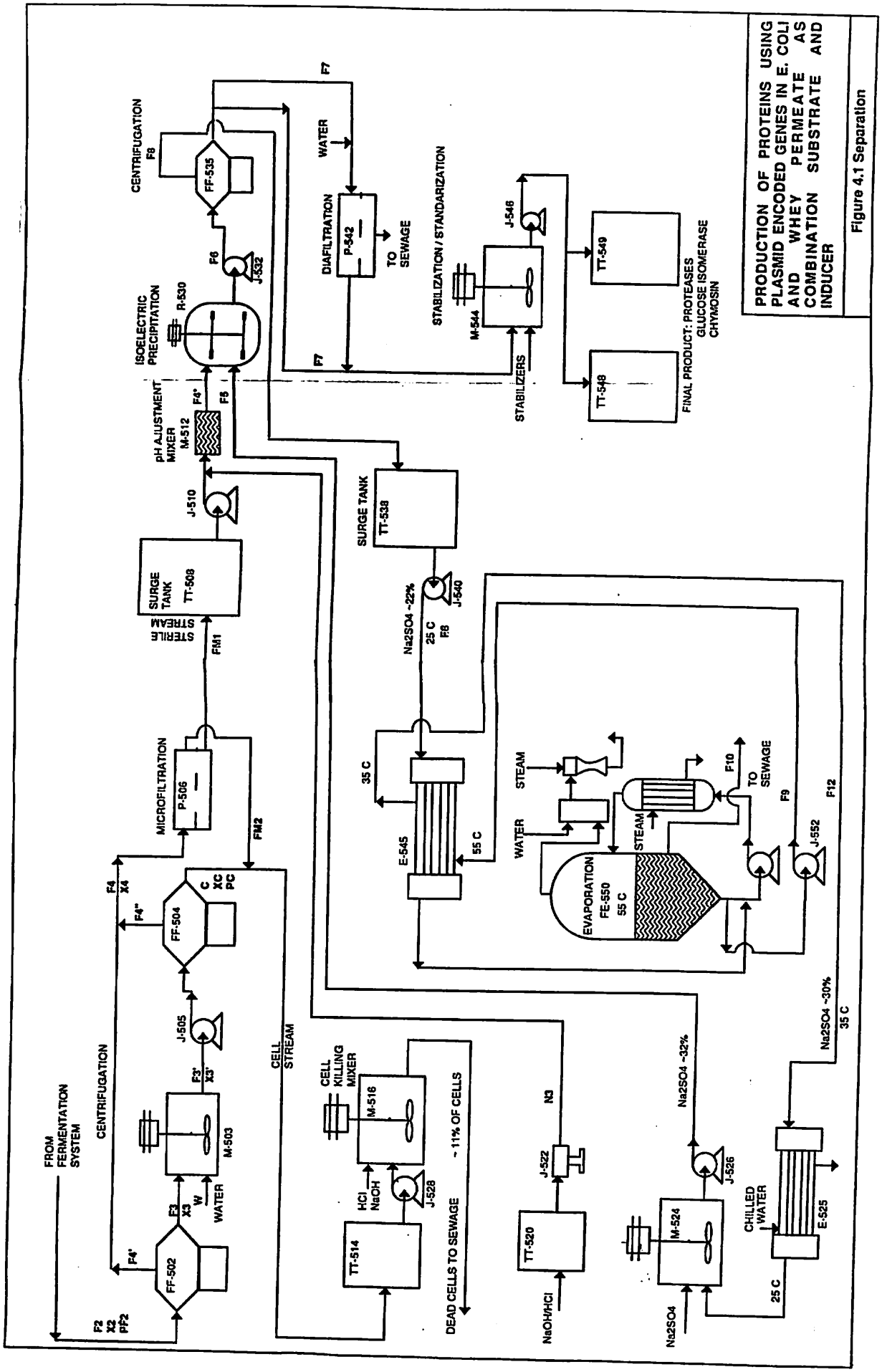


Table 4.1 Streams' Flows and Compositions
(Separation System)
(Scale: 0.50 Million Pounds of Permeate/Day)

Stream	β-lactamase (g/L)	Cell Concen. (g/L)	Na_2SO_4 (% -w/w-)	Temp. (° C)	Flow (kg/hr)
C	~0	141	0	25	1221
C+FM2	~0	111	0	25	1535
F2	4.74	25.31	0	25	6759
F3	-	141	0	25	1221
F3'	-	87	0	25	1974
F4	4.44	-	0	25	6291
F4*	4.44	0	0	25	5977
F5	0	0	32.0	25	12592
F6	1.61	0	21.7	25	18569
F7	1115	0	-	25	28
F8	0	0	21.7	25	18541
F9	0	0	30.0	55	12232
F10	0	0	30	55	5949
F12	0	0	30	35	12232
FM1	4.44	0	0	25	5977
FM2	-	-	0	25	315
N3	0	0	0	25	0.076
W	0	0	0	0	754

- Not calculated.

Precipitation is performed continuously by adding a saturated solution (F5) of Na2SO4 (figure 4.1). "Aging" of the precipitated protein is recommended to increase its recuperation efficiency[35,36]. In this plant, it is achieved by proper selection of the residence time and agitation (shear) in the reactor. Current precipitation theories does not allow the proper prediction of those quantities. Rules of thumb from water treatment applications are recommended for such purposes[37].

The centrifugation of the precipitated proteins produces an enzyme sludge with Na2SO4 as impurity (F7 steram in figure 4.1). For proteases, (because their major application is as detergent ingredient), it does not represent any problem. However, for food processing enzymes (glucose isomerase and calf rennet) the salts

must be taken out. This is an application for which diafiltration has been recommended.

FDA regulations prevent the release of recombinant DNA microorganisms to the environment. Cell killing is performed by keeping E. coli at pH 3 for four hours. It is assumed that dead cells are discarded into the sewage. If that is environmentally unacceptable, digestion to biogas is regarded as an alternative. The design of that process was out of the scope of this project.

Evaporation with force circulation evaporators was selected to concentrated the sodium sulfate stream. Such evaporator perform well under high scaling conditions. Heat integration was included in the design.

To stabilize the solution, sodium benzoate is employed. Glucose isomerase is sold principally in immobilized pellets, and part of the proteases is commercialized in dust free powder. Information regarding the procedures utilized for converting the liquid enzymes to those commercial forms is scarce. For such reason, liquid was assumed as the final form of all the enzymes produced in the plant.

5. Profitability and Sensitivity Analysis

Table 5.1 present the assumptions made for the profitability analysis:

Table 5.1 Profitability Analysis Assumptions

World Market*		
Bacillus Proteases	2	Millions kg/year
Glucose Isomerase	0.125	"
Calf Rennet	0.0138	"
Selling Prices*		
Bacillus Proteases	100	$/kg of pure enzyme
Glucose Isomerase	250	"
Calf Rennet	5000	"
Production Time*		
Bacillus Proteases	65	% of total operating time
Glucose Isomerase	30	"
Calf Rennet	5	"
Contamination Losses*	2	% of enzymes produced
Plant Operating Life	10	Years

* Independent variables in the spreadsheet. Any of them can be changed to obtain new results.

The enzymes' world markets are given for 1991. Although they increase at a rate of 10 to 15% per year[38], they were assumed constant for the whole operating life of the plant. This is very conservative, but to certain extend, it accounts for unpredictable

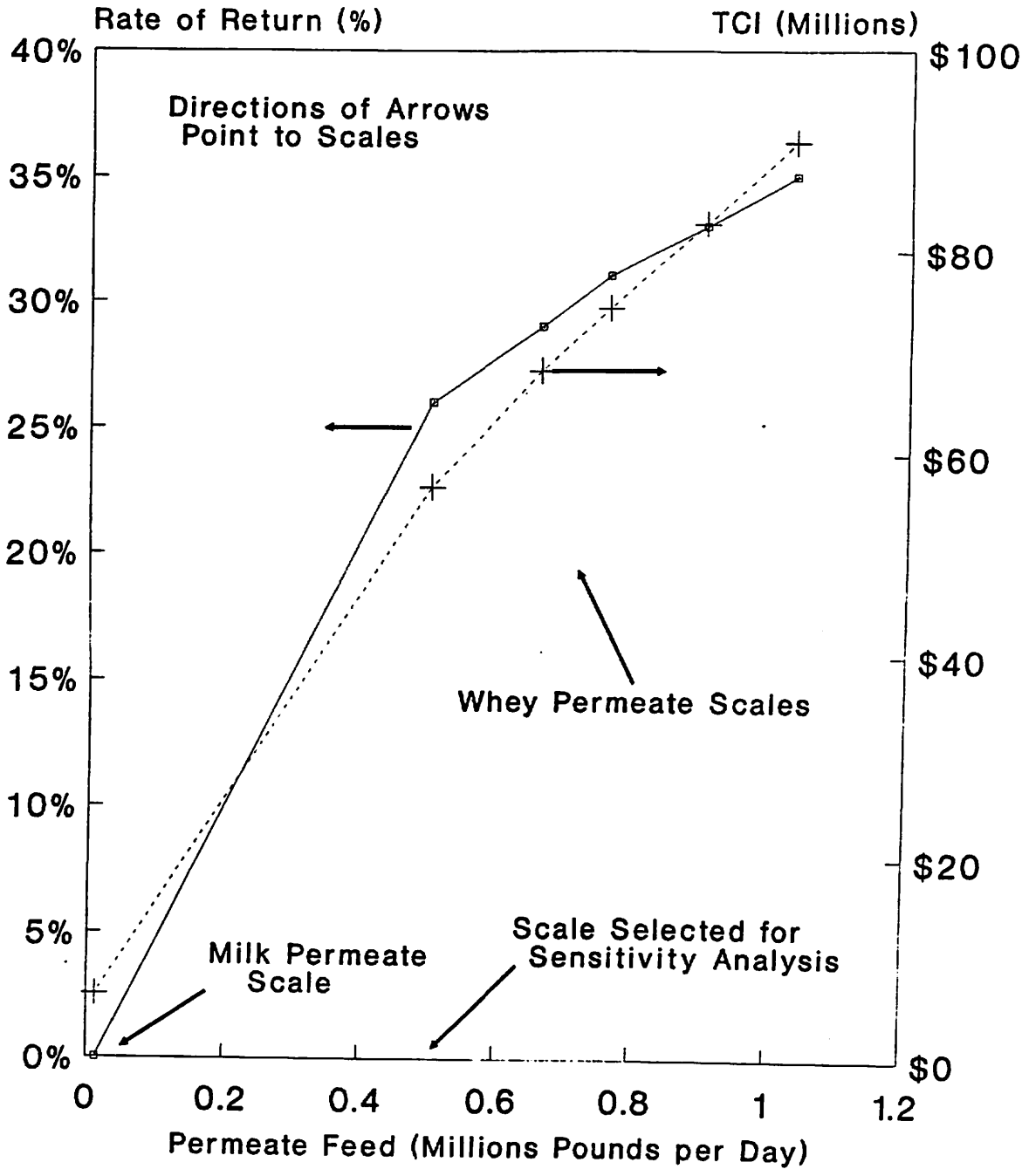


Figure 5.1 Enzymes' Production Profitability Analysis

The whey permeate scales show rates of return form 26 to 35%, which are considered very good. Figure 5.1 shows that the Total Capital Investment (TCI) is almost a linear function of the permeate feed. Such behavior was expected.

When the world market share is analyzed (table 5.2), a limitation shows up: the larger scales are not reasonable because the assumption that a plant would gain more that 50% of the world market of an enzyme, is very weak. Sensibility analysis was performed on the smallest whey permeate scale (0.5 million pound of permeate per day). It is not shown here.

The disposal revenues represent the difference between the savings obtained by not putting permeate into land or sewage (US$ 3/1000 gal)[39], and the cost of disposing brackish water (US$ 2/1000 gal)[40] (from the resins regeneration), dead cells (US$5/1000 gal)[41], and sodium sulfate (US$ 3/1000 gal)[42].

The total world whey production in 1987 was estimated at 100 millions metric tons[43]. It growths at 2% per year[44] Based on that, the 1991 world whey production is close to 108 millions metric tons. In 1982, the US share of such production was 17% and 45% of that was discarded[45]. Assuming the same percentages for 1982 and 1991, the US would be wasting close to 8 million metric tons of whey per year. The largest plant scale is 1.03 million pound per day. It represents less that 2% of the total whey disposed in the US and would be satisfying close to 62 and 93% of the world market of glucose isomerase and calf rennet. This is not reasonable. Consequently, under the present conditions, the CES can not be claimed as a viable solution to solve the whey disposal problems

in the US. However, this situation may change if markets for high volume proteins were found in the future.

6. Conclusions.

- Commercial enzyme production utilizing whey permeate and the Cornell Excretion System (CES) is profitable for processing plants with scales greater than 0.5 million pounds of permeate per day, and for smaller operations (0.013 million pounds per day) producing calf rennet only.

- Under current conditions, large scale operations (0.66 to 1.03 million pounds of permeate per day) are not feasible, because there is not enough enzyme market for their production.

- If new markets for high volume proteins does not appear in the future, the CES can not be claimed as a partial solution to the whey disposal problems in the US.

7. Recommendations.

- Because of academic and economic incentives, it is advised that the CES be experimentally tested with lactose from whey permeate as inducer.

8. Acknowledgments.

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