December 2009 Volume 3 Issue 4

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 3(4), 2009 [283-293]

Bioreactor design for the production of recombinant human therapeutic protein expressed in *Pichia pastoris* - A theoretical novel approach

Swaminathan Detchanamurthy[1]*, Salai Madhumathi Arasi Parkunan[2]

[1]Department of Chemical and Process Engineering, University of Canterbury, Private Bag: 4800, Christchurch-8140, (NEW ZEALAND)

[2]Department of Biotechnology, Sri Venkateswara College of Engineering Sriperumbudur, Tamilnadu-602105, (INDIA)

E-mail : swami_biochem@yahoo.co.uk

Received: 18th September, 2009 ; Accepted: 28th September, 2009

ABSTRACT

Bioreactors are the heart of any bioprocess operations. Currently there is a growing need for an efficient production of recombinant human therapeutic proteins worldwide. Most of the biotechnology based industries prefer *Pichia pastoris* as a host system for producing recombinant human therapeutic proteins because of the high cell density and product yield obtained from this particular species. This paper describes the bioreactor design procedure for producing 10kg yr^{-1} of recombinant human follicle stimulating hormone through *Pichia* pastoris. The protein used in our study is only a model for this work. Complete material and energy balance calculations were done in this study which is prerequisite for design calculations. It was calculated that for producing 10kg yr^{-1} of our model protein, a 26500L working volume bioreactor is required. Design calculations were carried out by fixing the $k_L a$ as 0.080 sec^{-1}. Mechanical design calculations were also performed in this work. The novel design calculation methods presented in this work here is very easy to learn and easy for the designers to design an efficient bioreactor for producing any proteins or enzymes in an industrial scale by only changing the few variables such as the empirical formula of the microbe and product of interest, operating conditions etc.

KEYWORDS

Bioreactor;
Pichia pastoris;
Therapeutic protein;
Scale up;
Material balance.

INTRODUCTION

Bioreactor refers to any device or system that supports a biologically active environment. Bioreactors are commonly cylindrical, ranging in size from liters to cubic meters, and are often made of stainless steel. On the basis of modes of operation, a bioreactor can be classified as batch, fed batch and continuous. Organisms growing in bioreactors may be suspended or immobilized. Bioreactor design is a relatively

FULL PAPER

complex engineering task, which is studied in the discipline of biochemical engineering. Under optimum conditions, the microorganisms or cells are able to perform their desired function with 100 percent rate of success. The bioreactor's environmental conditions like gas flow rates, temperature, pH and dissolved oxygen levels, and agitation speed/circulation rate need to be closely monitored and controlled[1]. Most industrial bioreactor manufacturers use vessels, sensors and a control system networked together. There are 2 different methods followed to design a large scale bioreactor, either by theoretical calculations or by any of a number of commercial simulations. Though the commercial simulations are faster and accurate they are very costlier for license[2]. Hence, in this work the theoretical approach for bioreactor design was used with more care in calculation accuracy.

The production of recombinant therapeutic proteins began to be developed more than 25 years ago[3,4] with nowadays more than 300 protein products on the market or in late clinical stages[5]. The vast majority of these proteins have been produced in microbial (e.g., *Escherichia coli*, *Saccharomyces cerevisiae*) or mammalian systems (mainly Chinese Hamster Ovary cells). While prokaryotic systems are clearly superior in terms of ease of handling and high product yields, eukaryotes like yeast, mammalian cell lines are favoured when correct post-translational modification of the protein target is necessary[5-8]. Though mammalian cells are higher eukaryotes, they face a big problem of contamination and huge downstream processing steps in industrial scale. *Pichia pastoris* is one of the methylotrophic yeasts which are of both academic and industrial interest for the past one decade. It can grow both in glucose and glycerol mediums but can able to synthesise or secret proteins only under methanol medium under the activation of alcohol oxidase AOX1 promoter. Expression of the AOX1 gene is tightly regulated and induced by methanol to high levels of protein synthesis which is virtually, absent in glucose or glycerol grown cells. Fermentation can be conducted over a wide pH range (3.0-6.0) at 30°C using *P.pastoris*[9]. In comparison to *Saccharomyces cerevisiae*, *Pichia* have an advantage in the glycosylation of secreted proteins because it will not hyperglycosylate. Both *Saccharomyces cerevisiae* and *Pichia pastoris* have a majority of N-linked glycosylation of the high mannose type. One of the drawbacks with Saccharomyces cerevisiae was that it did not have a strong inducible promoter. *Pichia pastoris* has a strong inducible promoter[10]. Considering the advantageous and wide industrial use of *Pichia pastoris* for the production of recombinant human therapeutic proteins, it has considered as a model organism in the present work for bioreactor design calculations. Moreover, we have considered human follicle stimulating hormone, a therapeutic protein which is used to treat human infertility as a model protein in our work. Our bioreactor design strategy can be employed for any protein or enzyme by replacing the empirical formula of the desired protein or enzyme in the calculation part. This case is applicable even for the micro organism selected in our work.

The method presented here is easy to learn, and offers a quick way to make preliminary estimates of the bioreactor design parameters.

METHOD

Material balance

The first step in a bioreactor design calculation is a material balance. Calculations were done initially for a shake flask fermentation conditions. The whole fermentation process was divided into 5 modes as shown in TABLE 1 & the material balance was done[10]. These 5 modes are already optimized for *Pichia pastoris* fermentation[10].

Batch mode

Considering glycerol as the carbon source and ammonium hydroxide as a nitrogen source for the initial growth of *Pichia pastoris*, and the empirical formula for *Pichia pastoris* (yeast) as $CH_{1.79}O_{0.5}N_{0.2}$ the following stoichiometric equation was developed[11].

$$C_3H_8O_3 + aO_2 + bNH_4OH \rightarrow cCH_{1.79}O_{0.5}N_{0.2} + dCO_2 + eH_2O$$

Here a, b, c, d and e are stoichiometric coefficients. The biomass yield for *Pichia pastoris* was taken as, $Y_{xs} = 0.4\ g\ g^{-1}$ and the percentage of ash content were taken as 8% in this calculation[11,12].

Calculation of stoichiometric co-efficients

$$c = 0.4 \times \frac{\text{Molecular weight of glycerol}}{\text{Molecular weight of biomass with ash}} \quad (1)$$

$$c = 0.4\frac{92}{26.73}$$

c = 1.376 mol biomass/mol glycerol

For calculating 'a', electron balance approach was used[11].

$$w\gamma_S - 4a = c\gamma_B \quad (2)$$

where w, γ_S and γ_B are number of carbon atoms in substrate, degree of reduction for substrate and degree of reduction for biomass.

w= 3

γ_S =[3×4 + 8×1 + 3×(-2)]/3 => 4.67],

γ_B =[1×4 + 1.79×1 + 0.5×(-2) + 0.2×(-3)]/1 => 4.19

Therefore, a= 2.06114 mol O_2/mol substrate

For calculating 'b', Nitrogen balance approach was used[11].

$$b = 0.20 \times c \quad (3)$$

b = 0.2752 mol NH_4OH/mol substrate

For calculating'd', Carbon balance approach was used[11].

1.376 + d= 3

d= 1.624 mol CO_2 / mol substrate

Finally for calculating 'e', Hydrogen balance approach was used[11].

8 + 4×b = 1.79 + 2×e

e = 3.319 mol H_2O/mol substrate

Hence the overall equation becomes,

$$C_3H_8O_3 + 2.06114O_2 + 0.2752NH_4OH \rightarrow 1.376CH_{1.79}O_{0.5}N_{0.2} + 1.624CO_2 + 3.319H_2O$$

OD_{600} value for *Pichia pastoris* is 42 once it reaches the exponential phase[10].

By correlation[13],

$$\text{Wet Cell Weight (WCW)} = OD_{600} \times 0.22 = 9.24\ gL^{-1} \quad (4)$$

Volume of basal salt medium taken= 1.5L

Volume of Trace Metal Solution (TMS) taken= 6.6mL

Volume of Inoculum taken= 150mL

Therefore, Total volume in the glycerol batch mode,

V = 1.657L

Therefore,

Total WCW for 1.657L = 9.24 g/L ×1.657L = 15.31g

= 15.31/26.73 = 0.5704mol

1mol glycerol yields 1.376mol biomass therefore, moles of glycerol required to yield

0.5704mol biomass = 0.4145mol glycerol

2.06114mol O_2 yields 1.376mol biomass therefore, moles of O_2 required to yield

0.5704mol biomass=0.8544mol O_2

0.2752mol NH_4OH yields 1.376mol biomass therefore, moles of NH_4OH required to yield

0.5704mol biomass =0.1141mol NH_4OH

Therefore, the overall stoichiometric equation can be rewritten as,

$$0.4145C_3H_8O_3 + 0.8544O_2 + 0.1141NH_4OH \rightarrow 0.5704CH_{1.79}O_{0.5}N_{0.2} + aCO_2 + bH_2O$$

By Carbon balance & Hydrogen balance, 'a' & 'b' values were calculated.

a= 0.6731 mol CO_2, b= 1.4327 mol H_2O

Therefore, overall equation at the end the batch mode can be rewritten as,

$$0.4145C_3H_8O_3 + 0.8544O_2 + 0.1141NH_4OH \rightarrow 0.5704CH_{1.79}O_{0.5}N_{0.2} + 0.6731CO_2 + 1.4327H_2O$$

Fed-batch mode-I

Fixed volume fed batch was considered for our calculations, where the limiting substrate is fed without diluting the culture. One of the advantage of this method is the culture volume can be maintained practically constant by feeding the growth limiting substrate in undiluted form and alternatively, the substrate can be added by dialysis without affecting the culture volume[11]. The following calculations were done to evaluate the parameters and stiochiometric coeffients in fed batch Mode I with glycerol as substrate.

Initial volume of fed batch,

V_0 (at the end of batch mode) =1.65L

Fed batch time, t_{fb} (from TABLE 1) =22.5 hr

Substrate volumetric flow rate,

F(from TABLE 1) =24mL$L^{-1}hr^{-1}$

Volume of trace metal solution added, V_{tms} =19.80mL

Fed batch volume, $V_{fb} = F \times V_0 \times t_{fb}$ = 891mL

Total volume at the end of fed batch,

$$V_T = V_0 + V_{tms} + V_{fb} = 2561mL \quad (5)$$

Mass flow rate of the substrate, G = 12g L^{-1} hr^{-1}

(**Note:** 100mL of 50% glycerol has 50 g glycerol; therefore 24mL of 50% glycerol has 12 g glycerol)

Volume of 50% glycerol added per liter of fermentation broth = 24mL L^{-1} hr^{-1}×22.5hr = 540mL L^{-1}

For 1.65L of fermentation broth, volume of 50% glycerol added= 540mL/L×1.65L= 891mL

(**Note:** 50% glycerol= 50g glycerol in 100mL of water)

FULL PAPER

Therefore, Amount of 50% glycerol required, S=(891×50)/100= 445.50g

Final amount of biomass formed at the end of fed batch-I,

$$X_t = X_0 + X_{fb} \quad (6)$$

where, X_0 is the amount of biomass formed during the batch mode & X_{fb} is the amount of biomass formed only at the glycerol fed batch mode

$$= (x_0 \times V_0) + (G \times Y_{xs} \times t_{fb}) \times V_t \quad (7)$$

$$= 291.84g$$

The specific growth rate for fed batch mode I was calculated from the following equation[11],

$$\mu = (G \times Y_{xs}) / x_0 \quad (8)$$

$$\mu = 0.52\ hr^{-1}$$

Therefore, stoichiometric equation for this fed batch mode is given by,

$$C_3H_8O_3 + aO_2 + bNH_4OH \rightarrow cCH_{1.79}O_{0.5}N_{0.2} + dCO_2 + eH_2O$$

The stoichiometric coefficients a, b, c, d and e were calculated by similar way as in Batch mode operation and the final stoichiometric equation for this mode was deduced to,

$$C_3H_8O_3 + 1.2639O_2 + 0.4274NH_4OH \rightarrow 2.137CH_{1.79}O_{0.5}N_{0.2} + 0.863CO_2 + 3.1558H_2O$$

Mass balance was carried out and tabulated in TABLE 2.

Fed-batch mode-II

In this fixed volume fed batch mode operation, methanol was used as a sole carbon source and ammonium hydroxide as a nitrogen source. Sine, protein expression commences in *Pichia pastoris* under methanol induction mode, the empirical formula for our model protein human follicle stimulating hormone was also considered in the product side of the stoichiometric equation. The empirical formula for the model protein was obtained from ProtParam™ bioinformatics tool. The following calculations were made to evaluate various parameters and stoichiometric coefficients for fed batch mode II.

Initial volume of fed batch,

V_0(at the end of fed batch mode I) = 2.561L

Fed batch time, t_{fb} (from TABLE 1) = 6 hr

Substrate volumetric flow rate,

F (from TABLE 1) = 10.9mL L^{-1} hr^{-1}

Volume of trace metal solution added, V_{tms} = 30.732mL

Fed batch volume,

$$V_{fb} = F \times V_0 \times t_{fb} \quad (9)$$

$$= 167.49mL$$

Total volume at the end of fed batch,

$V_T = V_0 + V_{tms} + V_{fb} = 2.7592L$

Mass flow rate of the substrate, G= 10.9g L^{-1} hr^{-1}

(**Note:** 100mL of 100% methanol has 100 g methanol; therefore 10.9mL of 100% methanol has 10.9g methanol)

Volume of 100% methanol added per liter of fermentation broth= 65.4mL L^{-1}

For 2.561L of fermentation broth, volume of 100% methanol added, = 65.4mL L^{-1} ×2.561L =167.49mL

(**Note:** 100% methanol =100g methanol in 100mL of water)

Therefore, Amount of 100% methanol required, S= 167.49g

Final amount of biomass formed at the end of fed batch-II,

$$X_t = X_0 + X_{fb} \quad (10)$$

where, X_0 is the initial amounts of biomass present at the start of fed batch-II & X_{fb} is the amount of biomass formed at the end of fed batch mode-II= 364.0207g

Amount of Protein formed at this Fed Batch Mode-II,

$$P = r_p \times t_{fb} \quad (11)$$

where r_p is the product formation rate which can be evaluated from the following relationship,

$$r_p = \mu \times X_0 \times Y_{px} \quad (12)$$

where μ is the specific growth rate which decreases with time and Y_{px} is the Protein yield w.r.t biomass. Specific growth rate can be calculated by the following relationship,

$$\mu = (G \times Y_{xs}) / x_0 \quad (13)$$

$$= 0.0383\ hr^{-1}$$

10mg/L was assumed as the expression level of rh-FSH[14] and used to calculate the protein yield, Y_{px} which was calculated as 7.1617×10^{-5}g g^{-1} for this mode of operation. Therefore, stoichiometric equation for this fed batch mode including the empirical formula for our model protein in the product side is given by,

$$CH_4O + aO_2 + bNH_4OH \rightarrow cCH_{1.79}O_{0.5}N_{0.2} + dCO_2 + eH_2O + fCH_{1.55}O_{0.27}N_{0.31}$$

The stoichiometric coefficients a, b, c, d, e and f were calculated and the final stoichiometric equation

for this mode was deduced to,

$CH_4O + 0.9983O_2 + 0.095793NH_4OH \rightarrow 0.4789CH_{1.79}O_{0.5}N_{0.2} + 0.52106CO_2 + 1.811H_2O + 4.12744\times10^{-5}\ CH_{1.55}O_{0.27}N_{0.31}$

Mass balance was carried out and tabulated in TABLE 3.

Fed-batch mode-III

The following calculations were made to evaluate various parameters and stoichiometric coefficients for fed batch mode III.

Initial volume of fed batch,

V_0 (at the end of fed batch mode II)= 2.7592 L

Fed batch time, t_{fb} (from TABLE 1)= 48 hr

Substrate volumetric flow rate,

F (from TABLE 1)= 15mL L^{-1} hr^{-1}

Volume of trace metal solution added, V_{tms} = 33.1104mL

Fed batch volume,

$$\mathbf{V_{fb} = F\times V_0\times t_{fb}} \quad \mathbf{(14)}$$

$$\mathbf{= 1986.624mL}$$

Total volume at the end of fed batch, V_T = 4.7789L

Mass flow rate of the substrate, G= 15g L^{-1} hr^{-1}

Volume of 100% methanol added per liter of fermentation broth= 720mL L^{-1}

For 2.7592 L of fermentation broth, volume of 100% methanol added= 1986.624mL

Therefore, Amount of 100% methanol required, S = 1986.624g

Final amount of biomass formed at the end of fed batch-III,

$X_t = X_0 + X_{fb}$

= 1740.341g

Amount of Protein formed at this Fed Batch Mode-III,

$$\mathbf{P = r_p\times t_{fb}} \quad \mathbf{(15)}$$

$$\mathbf{\mu = 0.04548\ hr^{-1}}$$

Y_{px} was calculated as 2.7564×10^{-5} g g^{-1} for this mode of operation.

Therefore, $r_p = (0.04548\times364.0207\times2.7564\times10^{-5})$

$\Rightarrow 4.5634\times10^{-4}$g hr^{-1}

Therefore, $P_T = P_0 + (r_p\times t_{fb})$

$\Rightarrow 0.02607 + (4.5634\times10^{-4}\times48)$

$\Rightarrow$ 0.04797g

Therefore, stoichiometric equation for this fed batch mode including the empirical formula for our model protein in the product side is given by,

$CH_4O + aO_2 + bNH_4OH \rightarrow cCH_{1.79}O_{0.5}N_{0.2} + dCO_2 + eH_2O + fCH_{1.55}O_{0.27}N_{0.31}$

The stoichiometric coefficients a, b, c, d, e and f were calculated and the final stoichiometric equation for this mode was deduced to,

$CH_4O + 0.99834O_2 + 0.09579NH_4OH \rightarrow 0.4789CH_{1.79}O_{0.5}\text{-}N_{0.2} + 0.5211CO_2 + 1.81085H_2O + 1.5892\times10^{-5}CH_{1.55}O_{0.27}N_{0.31}$

Mass balance was carried out and tabulated in TABLE 4.

Fed-batch mode-IV

The following calculations were made to evaluate various parameters and stoichiometric coefficients for this final fed batch mode IV.

Initial volume of fed batch,

V_0 (at the end of fed batch mode III) = 4.7789L

Fed batch time, t_{fb} (from TABLE 1) = 44 hr

Substrate volumetric flow rate,

F (from TABLE 1) = 2mL L^{-1} hr^{-1}

Volume of trace metal solution added, V_{tms} = 57.35mL

Fed batch volume, V_{fb} =420.5432mL

Total volume at the end of fed batch, V_T = 5.257L

Mass flow rate of the substrate, G = 2g L^{-1} hr^{-1}

Volume of 100% methanol added per liter of fermentation broth = 88mL L^{-1}

For 4.7789 L of fermentation broth, volume of 100% methanol added = 420.5432mL

Therefore, Amount of 100% methanol required,

S = 420.5432g

Final amount of biomass formed at the end of fed batch-IV,

$X_t = X_0 + X_{fb}$=1925.391g

Amount of Protein formed at this Fed Batch-IV (Methanol), $P = r_p\times t_{fb}$

μ = 0.0022 hr^{-1}

Y_{px} was calculated as 2.7303×10^{-5}g g^{-1} for this mode of operation.

Therefore, $r_p = (0.0022\times1740.341\times2.7303\times10^{-5})$

$\Rightarrow 1.0454\times10^{-4}$ g hr^{-1}

Therefore, $P_T = P_0 + (r_p\times t_{fb})$

$\Rightarrow 0.04797+(1.0454\times10^{-4}\times44)$

$\Rightarrow$ 0.05257g

Therefore, stoichiometric equation for this fed batch mode including the empirical formula for our model pro-

FULL PAPER

tein in the product side is given by,

$CH_4O + a\ O_2 + b\ NH_4OH \rightarrow cCH_{1.79}O_{0.5}N_{0.2} + dCO_2 + eH_2O + fCH_{1.55}O_{0.27}N_{0.31}$

The stoichiometric coefficients a, b, c, d, e and f were calculated and the final stoichiometric equation for this mode was deduced to,

$CH_4O + 0.99834O_2 + 0.095785NH_4OH \rightarrow 0.4789CH_{1.79}O_{0.5}N_{0.2} + 0.5211CO_2 + 1.81083H_2O + 1.57352 \times 10^{-5}CH_{1.55}O_{0.27}N_{0.31}$

Mass balance was carried out and tabulated in TABLE 5.

Therefore,

Total amount of biomass formed = 1925.391g

Total amount of protein produced in all the 3 methanol fed batch = 0.05257g

Determination of bioreactor volume

In-order to produce 10kg yr^{-1} of our model protein, the volume of bioreactor to be used has to be calculated. For calculating the bioreactor volume, the following method was adopted ASM manual of industrial microbiology and biotechnology[15].

Targeted protein per year = 10kg yr^{-1}

Recovery factor[9] = 75% (Since extracellular)

Therefore, required amount of annual target-protein to be produced = (10/0.75) =13.333 kg yr^{-1}

Fraction of target protein present in the-secreted protein[9]= 90%

Therefore,

Total protein to be produced = (13.333/0.9)

= 14.814 kg yr^{-1}

To run one fermenter cycle, time taken= Fermentation time + Downtime= 141 hrs+10 hrs =151 hr

Therefore no. of cycles to be run per year

= (365×24)/151=58 cycles yr^{-1}

To produce 0.05257 g of protein per cycle-amount of biomass needed is = 1925.391g

Therefore to produce 14814 g of protein per 58 cycles, (i.e., 255.414 g $cycle^{-1}$)-amount of biomass needed is

= 9354.61 kg biomass/cycle

= 542567.38 kg biomass yr^{-1}

Final wet cell concentration = (1925.391 g / 5.257 L)

= 366.253 g L^{-1}

= 0.366253 kg L^{-1}

Total volume of broth

=(542567.38kg/year)/ (Final wet cell conc.)

=1481400.507L yr^{-1}

Assume one bioreactor

Working volume =(1481400.507L yr^{-1})/58 cycles

= 25541.39 L

Actual volume

= (25541.39 L)/0.7[30% head space is accounted]

= 36487.7L =36500 L (approx)

Seed bioreactor volume = 36500×0.05

(Where 0.05 is volume ratio-Constant) = 1825L

Pre-seed bioreactor volume = 1825×0.05 = 91.25L

Inoculum development bioreactor volume

= 91.25×0.05 = 4.563 L

Bioreactor design

The following datas were used as a standard data for the design calculations[10,16].

Operating Temperature, T=30°C

Viscosity, $\mu = 25 \times 10^{-3}$ kg m^{-1} sec^{-1}

Density, ρ =1030 kg m^{-3}

Bioreactor Volume, V =36,500 L (or) 36.5 m^3

Y_{O2X} =2.4145 gg^{-1}

Bioreactor design was divided into following 12 steps

Step 1

OTR = OUR

i.e., $k_La\ (C^*-C) = \mu \times Y_{O2X} \times x_t$

Here μ is the specific growth rate. This value is taken as 0.0022 hr^{-1} (which is lowest among other μ values in the series of both batch and fed batch fermentations).

k_La-Volumetric oxygen transfer co-efft in sec^{-1}

C*-Equilibrium concentration of oxygen in mg L^{-1}

C-Bulk concentration of oxygen in mg L^{-1}

C*was calculated from the below correlation,

$C^* = 14.161 - 0.3943\ T + 0.007714\ T^2 - 0.0000646\ T^3$

$C^* = 14.161 - 0.3943\ (30) + 0.007714\ (30)^2 - 0.0000646\ (30)^3$

C*= 7.5304 mg/L

The value of C is 10% of C*[11]. Hence it was calculated as,

C = 0.75304 mg L^{-1}

x_t is the final biomass concentration

$x_t = X_t / V_f$

x_t = 9354.61/25541.39

x_t = 366252.97 mg L^{-1}

Therefore,

$k_La = (\mu \times Y_{O2X} \times x_t)/(C^* - C)$
$k_La = (0.0022 \times 2.4145 \times 366252.97)/6.7774$
$k_La = 287.06\ hr^{-1}$
$k_La = 0.080\ sec^{-1}$

Step 2

$V = (\pi/4) \times (D_T)^2 \times H_L$
Where, V is the bioreactor volume.
D_T is the bioreactor diameter.
H_L is the height of liquid in the bioreactor.
Generally in bioreactor, diameter will be equal to liquid height[16].
Therefore, $V = (\pi/4) \times (D_T)^3$
$(D_T)^3 = 36.5 \times (4/\pi)$
$D_T = 3.5953$ m
Usually the ratio of tank diameter to the impeller diameter should be 3, $(D_T/D_i = 3)$[17]
where D_i is the impeller diameter of the bioreactor
$D_i = 3.5953/3 = 1.1984$ m
Tank diameter was rounded off to $D_T = 3.3$ m as it should be lesser than the calculated value[17]. Similarly, the impeller diameter was selected as 1.3 m as it should be greater than the calculated value[17]. Trail and errors were done to obtain these values.
Therefore, $H_L = (V/D_T^2) \times (4/\pi)$
$H_L = 4.3$ m
Hence, $(D_T / D_i)^* = (3.3/ 1.3) = 2.54$
& $(H_L / D_i)^* = (4.3/ 1.3) = 3.308$

Step 3

Agitator speed N was selected as 228rpm on trail and error basis[17] such that, $\pi N D_i$ should be greater than 2.5 m s^{-1}
i.e., N = 3.8rps

Step 4

Gas velocity, v_g should be less than 125m/hr also, the calculated Q_g should be less than $0.6 \times (1.3)^5 \times (3.8)^2/(3.3)^{1.5}$ therefore, it was selected as 124 m/hr after trail and error.
$v_g = 0.0344$ m s^{-1}
Volumetric gas flow rate, $Q_g = v_g \times A$
$Q_g = 0.0344 \times (\pi / 4) \times (D_T)^2$
$Q_g = 0.2942\ m^3\ s^{-1}$
Therefore, vvm = Q_g (in $m^3 min^{-1}$)/V
vvm = 17.676 / 36.5
vvm = 0.4843

Step 5

Calculation of ungassed power:
$N_{Re} = \delta \times N \times (D_i)^2 / \mu$
$N_{Re} = 1030 \times 3.8 \times (1.3)^2 / 25 \times 10^{-3}$
$N_{Re} = 2.65 \times 10^5$
From figure 1, N_P Vs N_{Re} by considering ruston turbine[18],
$N_P = 8$
But $N_P = P/ (\rho \times N^3 \times (D_i)^5)$
Hence, $P = N_p \times (\rho \times N^3 \times (D_i)^5)$
$P = 8 \times 1030 \times (3.8)^3 \times (1.3)^5$
P = 1678783.774 Watts

Step 6

Correction for geometry

$F_c = [(D_T / D_i)^* \times (H_L/D_i)^*]^{1/2} / [(D_T/D_i) \times (H_L/D_i)]^{1/2}$
$F_c = [\ 2.54 \times 3.308\ ]^{1/2} / [\ 3 \times 3\ ]^{1/2}$
$F_c = 0.97$
Therefore, corrected power,
$P_c = 0.97 \times P$
$P_c = 0.97 \times 1678783.774$
$P_c = 1628420.261$ W

Step 7

Correction for number of impellers

$[(H_L - D_i)/D_i] > N_i > [(H_L - 2 \times D_i)/D_i]$
$2.31 > N_i > 1.31$
Therefore, $N_i = 2$ (Impellers)
Hence ungassed power, $P_{un} = 2 \times P_c$
$P_{un} = 21628420.261$
$P_{un} = 3256840.522$ W

Step 8

Correction for aeration

Flow number, $N_Q = (Q_g)/(N \times (D_i)^3)$
$N_Q = 0.2942 / (3.8 \times (1.3)^3)$
$N_Q = 3.524 \times 10^{-2}$
From figure 2, (P_g/P) Vs N_Q by considering flat blade turbine[18],
$(P_g/P_{un}) = 0.78$
Therefore, $P_g = 0.78 \times P_{un}$
$P_g = 2540335.607$ W

Step 9

Determination of k_La

$k_La = [0.0333 / (D)^4] \times (P_g/V)^{0.541} \times (Q_g)^{0.541/sqrt(D)}$

FULL PAPER

Here D is D_T

$k_La = 2.81\times10^{-4} \times416.72 \times0.6946$

$k_La = 0.081\ sec^{-1}$

Since the calculated k_La and the target k_La are more or less equal, the parameters which were assumed and calculated were accepted and the design is perfect. Moreover, 10% deviation is accepted usually between calculated & target k_La[17].

Step 10

Gas holdup & head space calculation

$\Phi = (V_T - V_L)/V_T = 1.8 \times(P_m)^{0.14} \times(v_g)^{0.75}$

$P_m = P_g/(\rho\times V)$

$P_m = 2540335.607/(1030 \times36.5)$

$P_m = 67.57\ W\ kg^{-1}$

Therefore, $[1-(V_L/V_T)] = 1.8 \times(67.57)^{0.14} \times(0.0344)^{0.75}$

$V_T = 49.31 m^3$

But, $V_T = (\pi/4)\times(D_T)^2 \times H_L^*$

$H_L^* = 49.31\times(4/\pi)/(3.3)^2$

$H_L^* = 5.77$ m

Allowing 10% head space, $H_L^{**} = 6.35$ m

Step 11

Calculation of heat duty: (Energy balance)

Heat released, $Q = -Q_{met} - Q_{shaft}$

$Q_{shaft} = P_g$

$Q_{shaft} = 2540335.607$ J/sec

$Q_{shaft} = 2540335.607\times3600 / 4.184$

$Q_{shaft} = 2185757.214\ kcal\ hr^{-1}$

$Q_{met} = (\mu \times Y_{O2X} \times x_t)$

Here maximum μ value (fed batch glycerol phase) was considered.

i.e. $\mu = 0.52\ hr^{-1}$

Therefore, $Q_{met} = (0.52\times2.4145\times366.253)$

$Q_{met} = 459.85\ g\ L^{-1}\ hr^{-1}$

$Q_{met} = 524516.41\ gmol\ hr^{-1}$

1 gmol of O_2 produces 460×10^3 J energy,

Therefore 524516.41 gmol hr^{-1} of O_2 would produce,

$= 2.413\times10^8\ kJ\ hr^{-1}$

$= 57672.08\ kcal\ hr^{-1}$

Therefore, $Q = -2185757.214 - 57672.08$

$Q = 2.243\times10^6\ kcal\ hr^{-1}$ (or) $2606.864\times10^3\ J\ s^{-1}$

To find amount of cooling water required

$Q = m\times C_p\times\Delta T$

Therefore, $m = Q/(C_p\times\Delta T)$

C_p of water = 75.4 J $mol^{-1}\ ^oC^{-1}$

$\Delta T = (T_2 - T_1)$, where T_2 is the water outlet temperature and T_1 is the water inlet temperature.

$\Delta T = (25-10) = 15^oC$

Hence, $= 2606.864\times10^3/(75.4\times15)$

m = 2305 mol water sec^{-1} (or) 41.5 kg water sec^{-1}.

Step 12

Mechanical design: (for bioreactor)

1) Cylindrical portion thickness,
   $t_c = (P_i\times D_T)[(2\times fJ) - (P_i)]$
   where, $P_i = 1.5\times10^5$ Pa (Design pressure)
   'f' for S.S @ $30^oC = 310\times10^6$ Pa
   Joint Efficiency, J = 0.85
   Therefore, $t_c = 0.94$ mm
2) Ellipsoidal head thickness,
   $t_e = (P_i\times D_T)/[(2\times fJ) - (0.2)\times(P_i)]$
   Therefore, $t_e = 0.94$ mm
3) Crown radius, $R_c = D_T = 3.3\times10^3$ mm
4) Knuckle radius, $R_k = 0.06\times R_c = 198$ mm
5) Support thickness (Skirt)=usually 20-40 mm
6) Nozzle design: Fermenter working volume,
   $V = 25.54139\ m^3$
   Flow rate=V/t
   't' is the time required to fill =45 min
   Therefore, flow rate $= (25.54139/45\times60)$
   $= 9.46\times10^{-3}\ m^3\ sec^{-1}$
   Nozzle area =flow rate/liquid velocity
   Liquid velocity $= 2\ m\ s^{-1}$
   Therefore, Nozzle area $= [9.46\times10^{-3}/2]$
   $= 4.73\times10^{-3}\ m^2$
   $(\pi/4)\times(D_N)^2 = 4.73\times10^{-3}$
   Hence D_N (Nozzle Diameter) = 77.60 mm
7) Cooling coil requirement: From heat duty calculation, $Q = 2606.864\times10^3$ J/sec But $Q = U\times A_1\times(\Delta T_L)$. Where, U is the overall heat transfer coefft = $850 Wm^{-2}K^{-1}$ & ΔT_L is the logarithmic mean temperature difference
   $= (\Delta T_1 - \Delta T_2)/\ln(\Delta T_1/\Delta T_2)$
   $\Delta T_1 = (30-10) = 20^oC$
   $\Delta T_2 = (30-25) = 5^oC$
   Hence $\Delta T_L = 10.82$
   Therefore, $A_1 = 283.44\ m^2$
   Available area $A_2 = \pi\times D_T\times(H_L^*)$
   $A_2 = 60\ m^2$
   $(\pi\times N_T\times D_T\times H_L) = (A_1 - A_2)$

here 'N_T' is the number of cooling coil turns.
$N_T = (283.44\text{-}60)/\pi\times3.3\times4.3) = 5$ turns.
The summary of overall calculated results were tabulated in TABLE 6 and based on the results, a bioreactor was draw with 1:50 scale (Figure 3).

RESULTS

Material balance results for all the modes of opera-

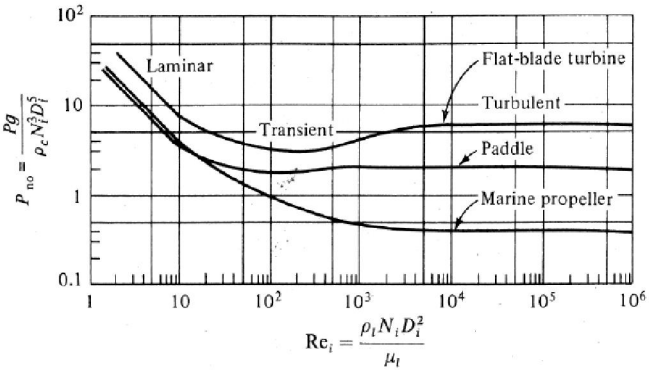
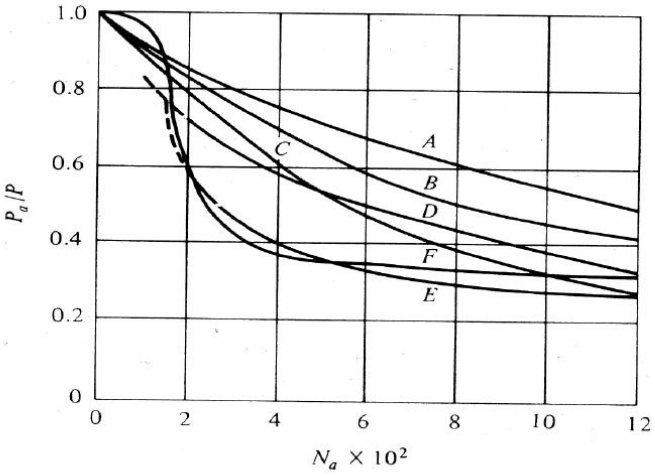


Figure 1 : Standard graph for N_P Vs N_{Re}

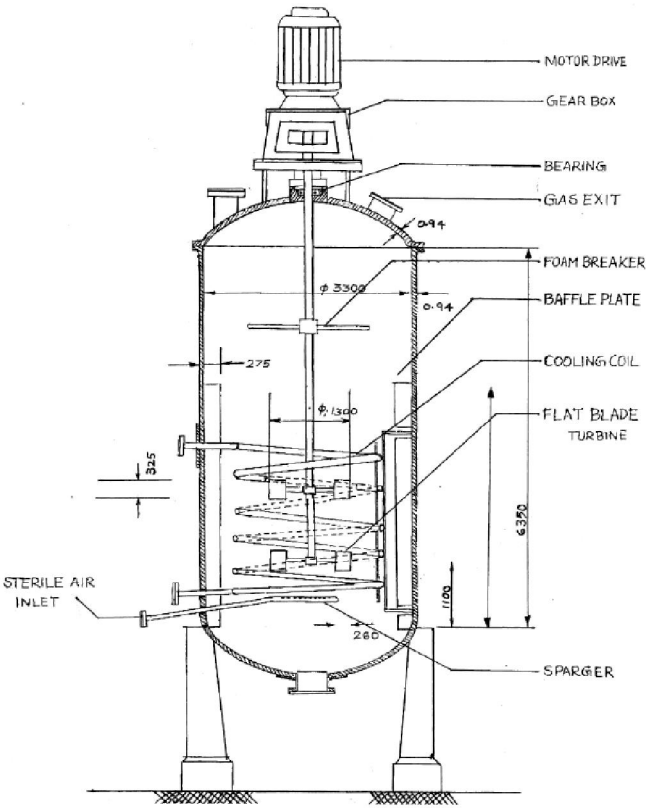


A-Flate blade turbines, B-Vanned disc (8 vanes), C-Vanned disc (6 vanes), D-Vanned disc (16 vanes), E-Vanned disc (4 vanes), F-Paddle

Figure 2 : Standard graph for (P_g/P) Vs N_Q

TABLE 1 : Standard fermentation conditions followed for *Pichia pastoris*

Time (Hrs)	Stage	Mode	Feed substance	Feed rate (mL/L/hr)
0-20	Growth	Batch	None	Not applicable
20-42.5	Growth	Fed-batch	50% Glycerol	24
42.5-43	Starvation	batch	None	Not applicable
43-49	Induction	Fed-batch	100% Methanol	10.9
49-97	Production	Fed-batch	100% Methanol	15
97-141	Production	Fed-batch	100% Methanol	2



(Scale-1:50, all demensions are in "mm")

Figure 3 : Bioreactor configuration with the calculated design parameters

TABLE 2 : Mass balance for Fed-Batch Mode-I

Mass in	Mass out
1 mol Glycerol = 92.0000g	1.3767 mol Biomass = 33.853g
2.0604 mol O_2 = 65.9328g	1.6233 mol CO_2 = 71.4252g
0.27534 mol NH_4OH = 9.6369g	3.456 mol H_2O = 62.208g
Total mass in = 167.57g	Total mass out = 167.49g

TABLE 3 : Mass balance for Fed-Batch Mode-II

Mass in	Mass out
1 mol Methanol = 32.0000g	0.4789 mol Biomass = 11.776g
0.9983 mol O_2 = 31.9456g	0.52106 mol CO_2 = 22.93g
0.095793 mol NH_4OH= 3.3528g	1.811 mol H_2O = 32.598g 4.12744×10^{-5}mol Protein=0.000917g
Total mass in = 67.2984g	Total mass out = 67.305g

TABLE 4 : Mass balance for Fed-batch mode-III

Mass in	Mass out
1 mol Methanol = 32.0000g	0.4789 mol Biomass = 11.776g
0.99834 mol O_2 = 31.9469g	0.5211 mol CO_2 = 22.93g
0.09579 mol NH_4OH = 3.35265g	1.81085 mol H_2O = 32.5953g 1.5892×10^{-5} mol Protein = 0.000353g
Total mass in = 67.29955g	Total mass out = 67.3017g

FULL PAPER

TABLE 5 : Mass balance for Fed-batch mode-IV

Mass in	Mass out
1 mol Methanol = 32.0000g	0.4789 mol Biomass = 11.776g
0.99834 mol O_2 = 31.9469g	0.5211 mol CO_2 = 22.93g
0.095785 mol NH_4OH = 3.3525g	1.81083 mol H_2O = 32.595g 1.57352×10^{-5} mol Protein = 0.000349g
Total mass in = 67.2994g	Total mass out = 67.30135g

TABLE 6 : Overall design calculation results

S.No	Design parameters	Values
1	H_L	4300 mm
2	H_L^*	5770 mm
3	H_L^{**}	6350 mm
4	D_T	3300 mm
5	D_i	1300 mm
6	N	3.8 rps
7	N_i	2
8	v_g	34.4 mm s^{-1}
9	Q_g	0.2942 m^3 s^{-1}
10	vvm	0.4843
11	Q	2606.864×10^3 J s^{-1}
12	m	41.5 kg water s^{-1}
13	t_c	0.94 mm
14	t_e	0.94 mm
15	R_c	3300 mm
16	R_k	198 mm
17	D_N	77.60 mm
18	N_T	5 turns
19	J (D_T/12)	275 mm
20	E (D_T/3)	1100 mm
21	W (D_i/5)	260 mm
22	L (D_i/4)	325 mm

tions gave a total amount of biomass synthesised as 1925.391g and the total amount of protein as 0.05257g. These values were used to calculate the bioreactor volume required to produce 10kg/year of our model protein (FSH). It was calculated that 25541.39L working volume bioreactor is required to synthesize the target quantity of the model protein. Furthermore, from the calculated working volume of the main bioreactor, the volumes of seed, pre-seed and inoculum bioreactors were also found out as 1825L, 91.25L and 4.563L respectively. From the working volume of the bioreactor, the actual volume was calculated as 36,500L. The actual volume was used in the design calculations. The k_La was calculated as 0.080 sec^{-1}. The design calculations were performed with various trail and errors in order to obtain the same k_La value which was one of the criteria for checking the considered design parameters were correct in this theoretical design procedure. Once, both the k_La values were nearly equal, the assumed design parameters were considered to fit best for the bioreactor and then mechanical design was carried out to evaluate the mechanical design parameters with different relationships available from literature. All the assumed parameters and calculated values were tabulated in TABLE 6.

CONCLUSION

The bioreactor design calculations used in this work is easy to learn and offers a quicker way for preliminary calculations of the bioreactor design. This method also enables the designers to scale up and optimize the bioprocess. By using this method the designer also escapes the costly simulation software license. Moreover, this work was completely carried out with plenty of assumptions since the design is theoretically done. So, if the exact experimental values are available for a particular protein or enzyme production using a particular microbe, then definitely the approach used in this work will benefit the designers to come up with exact design parameters.

REFERENCES

[1] L.Eva Decker, Ralf Reski; Bioprocess Biosyst.Eng., **31**,3-9 **(2008)**.

[2] Jolius Gimbun, A.B.Dayang Radiah, T.G.Chuah; Food Engineering, **64**, 277-283 **(2004)**.

[3] D.V.Goeddel, D.G.Kleid, F.Bolivar, H.L.Heyneker, D.G.Yansura, R.Crea, T.Hirose, A.Kraszewski, K.Itakura, A.D.Riggs; Proc.Natl.Acad.Sci.USA, **76**,106-110 **(1979)**.

[4] J.A.Martial, R.A.Hallewell, J.D.Baxter, H.M.Goodman; Science, **205**, 602-607 **(1979)**.

[5] F.R.Schmidt; Appl.Microbiol.Biotechnol., **65**, 363-372 **(2004)**.

[6] D.C.Andersen, L.Krummen; Curr.Opin.Biotechnol., **13**,117-123 **(2002)**.

[7] M.Butler; Appl.Microbiol.Biotechnol., **68**, 283-291 **(2005)**.

[8] G.Walsh, R.Jefferis; Nat.Biotechnol., **24**,1241-1252 **(2006)**.
[9] J.M.Cregg; Biotechnology, **11**, 905-910 **(1993)**.
[10] Y.Chen, J.Krol, J.Cino, D.Freedman, C.White, E.Komives; Journal of Chemical & Biotechnology, **67**, 143-148 **(1996)**.
[11] M.Pauline Doran; Bioprocess Engineering Principles, Academic press, **(1998)**.
[12] Chandrasekhar Gurramkonda, Ahmad Adnan, Thomas Gäbel, Heinrich Lünsdorf, Anton Ross, Satish Kumar Nemani, Sathyamangalam Swaminathan, Navin Khanna, Ursula Rinas; Microb.Cell Fact., **8**, 13-20 **(2009)**.
[13] Wenhui Zhang, Chih-Ping Liu, Mehmet Inan, M.Michael Meagher; J.Ind.Microbiol.Biotechnol., **31**, 330-334 **(2004)**.
[14] Fabien Richard; Journal of Biochemical and Biophysical Research Communications, **245**, 847-852 **(1998)**.
[15] W.Harvey Blanch, S.Douglas Clark; Biochemical Engineering, Marcel Dekker Inc., **(1996)**.
[16] C.Michael Flickinger, W.Stephen Drew; Encyclopedia of Bioprocess Technology, Fermentation, Biocatalysis and Bioseparation, Willey publications, **5**, **(1999)**.
[17] S.J.Hall, P.F.Stanbury, A.Whitaker; Principles of fermentation technology, Oxford Publication, **(1999)**.
[18] E.James Bailey, F.David Ollis; 'Biochemical Engineering Fundamentals', McGraw-Hill, Second Edition, **(1986)**.