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REVIEW

Bioprocess Optimization - a Challenge

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A biological process is a complex interaction between the cell and it's environment. It denotes an actual series of operations and interactions of living and non living matter. Dynamics and efficiency of a microbial process can be manipulated by a choice of physical, chemical and biological parameters. Optimization of a bioprocess requires knowledge of general microbiology, biochemistry, physiology, mathematics and computer applications. Figure 1 represents a simplified summary of interactions between "bioprocess optimization" and other science branches.

Optimization techniques are broadly classified into two categories: Statistical and non - statistical. Statistical techniques are more scientific and require rigorous mathematical manipulations. This technique may not be applicable to all systems. In such cases, we resort to non statistical techniques where the experimental points are designed to approach an optimum.

What is optimization and how is it done?

The set of values of input variables which result in the most desirable response value are called the "set of optimum conditions". The process of achieving these conditions is called "optimization". A "process analysis" approach is usually followed for any optimization. This is a basic method for description of a complex phenomenon and interaction among observed variables in the process under study. The unified analysis of a process determines the strategy for process optimization. The general steps in the optimization procedure are:

- Identification of all the variables which influence the process
- Screening of variables that have no significant effect on the process and choosing the most important ones.
- Selecting the detailed optimization strategy (statistical or non-statistical) depending upon variables and requirements.
- Mathematical/statistical analysis of the results obtained to determine the optimum points.
- Verification of the optimum points to give best results.

Statistical Optimization Techniques

A biological process is influenced by a variety of parameters that differ widely in nature. Also, in many cases, there is a complex interaction among these variables affecting the process. The classical method of studying one parameter at a time is highly inefficient and also needs very extensive experimental work. Sometimes, when the near optimum values are not clearly defined, it may not be possible to achieve an optimum by conventional techniques. To avoid these problems, statistical optimization is employed. Generally, statistical optimization involves the following steps (5):

- Design of experiments that will yield adequate and reliable measurements of the response.
- Detailed analysis of the results obtained to formulate adequate mathematical model (s).
- Check for fits of these models with experimental data using suitable hypotheses.

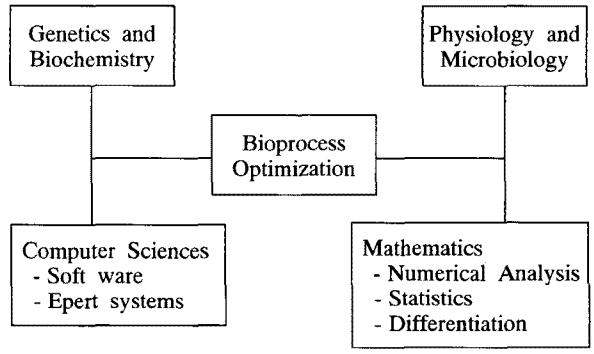

Fig. 1. Summary of interactions between bioprocess optimization and other science branches.

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• Verification of the optimum points obtained.

Two different types of design in this context, viz., first order and second order, were practiced in authors' laboratory. From the first order design the screening of effective variables were obtained to get the medium composition for β-1,3 glucanase (13) and CMCase production (10). The design employed in first order was Plackett-Burman design. The objective stated by this design was to obtain design that can estimate all main effects with maximum precision possible for N=k+1, where k is number of variables.

From the screened variables, the optimization of media constituents were done using the central composite design (12, 13). The interaction effects among the variables can be studied using second order design. The advantage of central composite design is that the effect of variables on axial points on axis of each design variable at a distance alpha from the design centre can be studied. The other second order design used was Box-Behnken which is a three-level incomplete factorial design formed by combining two-level factorial designs with balanced incomplete block designs (BIBD) in a particular manner.

The most commonly used statistical technique in bioprocess optimization is the response surface methodology (RSM). Response surface experiments attempt to identify the response of a system as a function of explanatory variables. Response surface methodology is most often used to determine the optimum response for the specific range of variable conditions. The interaction among the possible influencing parameters can be evaluated with limited number of well planned experiments. The wide applicability of statistical optimization will be evident from the case studies discussed below.

Other examples are given chronologically:

RSM was used for simultaneous optimization of temperature and initial pH in both surface and submerged-cultures of *Trichoderma harzianum* for the production of β-1,3-glucanase and to study the efficiency of the enzyme obtained under different conditions in generation of protoplasts from *Trichoderma reesei* mycelia as mentioned in Table 1 (13). Similar studies were made for endoglucanase production.

Table 1. Optimum levels of initial pH and temperature for production of β-1,3-glucanase and carboxymethyl cellulase.

Variables culture	Range	Optimum level			
		Surface culture		Submerged	
		β-1,3-glucanase	CMCase	β-1,3-glucanase	CMCase
Temperature (°C)	25~35	30.7	29.02	30.3	30.40
pH	4.0~6.0	4.7	4.85	4.7	4.86

Ethanol production by *Zymomonas mobilis* was studied by using different concentrations of glucose. A glucose concentration of 50~150 g/l showed the highest yield. The effect of inoculum age, yeast extract and peptone in the step feeding system was studied. A suitable combination of yeast extract and peptone was selected on the basis of the central composite design (CCD) procedure (11). A 2^2-factorial central composite design (CCD) was used for the above procedures. A 2^4-factorial CCD was used for the optimization of carbon and nitrogen sources for the production of extracellular chitinase by *T. harzianum* (Table 2). To optimize initial pH and temperature for the above system, a 2^2-factorial design and a similar design was used to optimize the biological parameters (4).

In order to obtain a single stage conversion of cellulose to ethanol, *T. reesei* QM9414, a hypercellulase producer was fused with *Saccharomyces cerevisiae*, a potent ethanol producer in our laboratory. Optimization of endoglucanase production, an enzyme responsible for the conversion of cellulosic materials to fermentable sugars, is a key factor in this case. The medium constituents consisting of 15 variables were optimized for endoglucanase production by *T. reesei* and by intergeneric fusant of *T. reesei/S. cerevisiae* using a Plackett-Burman design to screen the variables that significantly influence enzyme production followed by optimization of the significant grouped variables using a 2^5-fractional factorial central composite design. The results are given in Tables 3 and 4 (10).

Analysis of plasmids responsible for penicillin

Table 2. Optimum level and range of carbon and nitrogen sources used for the production of extracellular chitinase.

Variables	Range	Optimum level
Chitin (g/l)	0~10.0	12.5
Ammonium sulphate (g/l)	0~2.8	4.2
Peptone (g/l)	0~2.0	0
Urea (g/l)	0~0.6	0
pH	4.6~7.4	5.6
Temperature (°C)	23.0~37.0	28.0
Slant age (h)	38~106	105.0
Inoculum age (h)	19~53	43.0

Table 3. Range and optimum levels of variables in central composite design.

Variables	Range (g/l)	Optimum level (g/l)
Phosphate#	0~15.364	15.36
Ammonium sulphate	0~ 3.500	1.74
Peptone	0~ 2.500	0
Tween 80	0~ 0.500	0
Minerals	0~ 0.0787	0.078

#Consisting of KH_2PO_4 and NaH_2PO_4 in the ratio of 1 : 3.4 as PO_4^{3-}.

Table 4. Endoglucanase production in optimized and unoptimized medium.

Medium	Endoglucanase (U)	
	T. reesei	*T.reesei/ S. cerevisiae* fusant
Unoptimized reference medium	0.693	0.098
Final optimized medium	7.901*	0.334**

*Obtained with 40 g/l of dried grass as carbon source.
**Obtained with 50 g/l of dried grass as carbon source.

amidase synthesis by *Escherichia coli* and stability of the enzyme were studied using a central composite design (3). Optimal concentrations of carbon and nitrogen sources for the biosynthesis of pectinase by *Aspergillus niger* were done using a 2^3-factorial central composite design with six star points. The components optimized were corn, ammonium sulphate and glucose. The pectinase activity was increased by about 40% after optimization. This study also provided information about the optimum C/N ratio as in Table 5 (7).

Critical factors affecting electrofusion of sphaeroplasts of *S. cerevisiae* and protoplasts of *T. reesei* for direct conversion of cellulose to ethanol were optimized using statistical techniques. Optimization was carried out within the framework of central composite design for three pulse variables - pulse voltage, pulse duration and number of pulses and three A.C parameters - A.C frequency, A.C field strength and duration. Two 2^3-factorial designs were used for the above studies. As electrofusion appeared to offer advantages over conventional PEG fusion more detailed assessment of physical parameters which were found to affect fusion efficiency were studied. The fusion frequency was about 3.6×10^{-3}, significantly higher than by PEG method (Lakshmi Prasanna and Panda, unpublished results). The optimum values for an electrode gap of 0.5 mm are given in Table 6.

A central composite design was used to optimize the parameters affecting generation of protoplasts from *Penicillium griseofulvum, T. reesei, Trichoderma harzianum*

Table 5. Optimum levels of C/N ratio and comparison of pectinase activity with unoptimized medium.

Component	Level before optimization (g/l)	Pectinase activity un-optimized medium (U)	Levels after optimization (g/l)	Pectinase activity using optimized medium (U)
Corn	10		20.97	
$(NH_4)_2SO_4$	7	1.3	8.42	1.86
Glucose	40		3.46	

Table 6. Optimal electrical parameters for electrofusion of sphaeroplasts of *Saccharomyces cerevisiae* and protoplasts of *Trichoderma reesei.*

AC parameters	Pulse parameters
AC Frequency: 1 MHz	DC field strength: 5 kV/cm
Field strength: 400 V/cm	Pulse duration: 25 sec
AC duration: 30 sec	No. of Pulses: 5 pulses at an interval of 10 secs

and *Nocardia orientalis*. First, individual optima were obtained. This was followed by defining the rectangular confidence region and distance function, minimizing the distance function and solving a set of non-linear equations to give a simultaneous optima. The parameter s studied were slant age, age of mycelia and contact time with the lytic enzyme. Responses studied were yield of protoplasts, viability and production of product. Multiresponse and multiregression analysis were performed on the data obtained to get a simultaneous optima and the results are given in Table 7 (Muralidhar and Panda; Gokul and Panda, unpublished results).

Preliminary screening of physiological parameters, viz., slant age, inoculum age and inoculum level and media constituents, viz., sucrose, yeast extract, K_2HPO_4, KCl, $NaNO_3$, $MgSO_4$ and $FeSO_4$ for griseofulvin production by *P. griseofulvum* were done using Plackett-Burman design. The parameters that predominantly affect the production were optimized using central composite design (Dasu and Panda, unpublished results).

A central composite design was used to optimize initial pH and temperature for the production of tartaric acid by *Gluconobacter suboxydans*. A 2^2-factorial design with four star points in two blocks was used (Chandrasekar *et al.*, 1997, unpublished results). To optimize the production of propionic acid by *Propionibacterium freudenreichii* NCIM 2111, a 2^3-factorial designs were used to optimize biological parameters such as age of slant, age of inoculum and inoculum level and a 2^2-factorial design was used to optimize physical parameters such as initial pH and temperature. A 2^4-factorial designs were used to optimize medium constituents (Balamurugan and Panda, unpublished results). Optimization of citric acid

Table 7. Simultaneous maximum for generation of protoplasts from *Penicillium griseofulvum.*

Parameter (h)	Range (h)	Simultaneous optimum (h)
Slant age	80~192	182
Mycelial age in liquid culture	10~60	16
Contact time with lytic enzyme	2~30	15.2

Table 8. Optimum values of pH and temperature for maximum enzyme stability.

Enzyme	Range studied		Optimum values		Coefficient of Regression
	pH	Temp. (°C)	pH	Temp. (°C)	
Poly-galacturanase	2.5~8.1	23~37	4.8	28	0.95
Pectinlyase	2.7~8.3	23~37	3.9	29	0.95
Polymethyl galacturanase	1.3~3.7	13~27	2.2	23.1	0.933

It was found that pectinlyase was more stable than the other two enzymes.

production from molasses by *A. niger* and from *n*-alkanes by *Candida lipolytica* was done using central composite design. In this work, 2^3-factorial design was used to optimize carbon, nitrogen and phosphate sources in the fermentation medium (Pazouki and Panda, unpublished results). The pH and thermal stability of pectolytic enzymes (namely, polygalacturonase, polymethyl galacturonase and pectinlyase) were studie d using response surface methodology. A 2^k-factorial central composite design is employed (Naidu and Panda, unpublished results).

The combined effect of pH and temperature on endoglucanase (CMCase) from two intergeneric fusants of *T. reesei/S. cerevisiae* was studied using response surface methodology. A 2^k-factorial central composite design was employed to study the effect. A comparision was made with endoglucanase (EG) of *T. reesei* (WT) and is given in Table 9 (Srinivas and Panda, unpublished results).

A Box-Behnken design was used to optimize paramters for the production of gluconic acid from cane molasses by *A. niger*. Statistical designs were used to optimize variables affecting immobilization of *A. niger* cells and to study the performance of whole cell bioreactors for gluconic acid production (9). Optimization of medium constituents for single-step direct bioconversion of cellulosic materials to ethanol by intergeneric fusants

Table 9. pH and temperature combinations for endoglucanase from fusants and *T. reesi.*

Enzyme	Range studied		Optimum values		Coefficient of Regression
	pH	Temp. (°C)	pH	Temp. (°C)	
EG from fusant M14	3.39~6.2	36~64	5.7	42	0.9858
EG from fusant M62	3.39~6.2	36~64	5.3	43	0.9795
EG from *T. reesei*	3.39~6.2	36~64	4.3	38	0.9256

The endoglucanase from the fusants (M14 and M62) were found to be more stable compared to endoglucanase of *T. reesei.*

Table 10. Optimized medium composition.

Component	Concentration
Cellulose (as dried grass)	10.00 g/l
KH_2PO_4	2.22 g/l
NaH_2PO_4	7.65 g/l
$MgSO_4 \cdot 7H_2O$	0.34 g/l
$(NH_4)_2SO_4$	1.60 g/l
Citric acid monohydrate	9.24 g/l
Tween 80	0.22 g/l
Urea	0.30 g/l
$FeSO_4 \cdot 7H_2O$	5.0 mg/l
$MnSO_4 \cdot 6H_2O$	1.6 mg/l
$ZnSO_4 \cdot 7H_2O$	1.4 mg/l
$CaCl_2 \cdot 2H_2O$	1.9 mg/l

of *T. reesei/S. cerevisiae* was done using the Box-Behnken design method. The combined effect of pH and temperature on the fermentation was analyzed by the central composite design method. Under optimal conditions, the fusant produced 0.17 g/l ethanol in 30 h as in Table 10 (12).

Non-statistical Optimization

Non-statistical Optimization techniques are employed when a set or a block of experiments as required by statistical techniques cannot be performed (such as optimization of variables in reactors). This method is useful when the effect of a particular variable is to be assessed.

A single parameter optimization was carried out to find the role of phenylacetic acid on the expression of penicillin amidase (PA) by *E. coli* cells. A multifactor non-statistical optimization technique was used to find the effect of pH, glutaraldehyde concentration and cell concentration on the extent of immoblization of penicilllin amidase associated with *E. coli* cells.The stability of penicilllin amidase in free and immoblized forms at various temperatures were studied using the same technique (1). The results are summarized in Table 11.

Independent single parameter optimization was carried out to find the effect of age of organism in slant, age of cells, dry cell weight, lytic enzyme level, temperature, concentration of phosphate buffer and osmotic stabilizer on the generation of sphaeroplasts from *S. cerevisiae* (6). The results are given in Table 12.

Process development for the production of xanthan by

Table 11. Effect of different parameters on stability of immobilized penicillin amidase.

Parameter	Range	Optimum activity (%)	Relative residual PA at optimum
pH	2.0~8.0	4.25	80
Glutaraldehyde conc. (%)	0~3	1.5	90
Cell conc. (g/l)	0~100	37.5	92

Table 12. Optimal parameters for generation of sphaeroplasts from *S. cerevisiae*.

Parameter	Range	Optimum value
Age of inoculum (days)	0~8	1.0
Age of cell (h)	0~60	20.0
Lytic enzyme conc. (ml/g dry wt of cell)	0~250	72.9
Dry cell wt (g)	0.05~0.3	0.1262
Time of contact (min)	0~30	25.0
Temperature (°C)	4~60	30.0
Phosphate buffer conc. (mM)	10~150	25.0
Osmotic stabilizer, KCl (M)	0.5~2.0	0.7

Table 13. Optimized parameters for xanthan production by *X. malvacearum*.

Parameter studied	Range studied	Optimum values	Xanthan (g/l)
Slant age	24~120 h	48 h	3.75
Inocullum level	5 to 20% (v/v)	10% (v/v)	3.75
Initial glucose conc. (batch)	10~80 g/l	20 g/l	4.05
Glucose conc. maintained in batch step feeding	10~80 g/l	10 g/l	6.4

Xanthomonas malvacearum was done by studying the effect of physiological parameters, viz., slant age, inoculum level and the effect of glucose on the fermentation. The results are summarized in Table 13 (Uma and Panda, 1997, unpublished results).

Gluconic acid fermentation by *A. niger* was investigated using untreated and treated Indian cane molasses. The yield of gluconic acid was found to be reduced using an untreated molasses medium compared to defined medium. Hence, molasses was subjected to various pretreatment techniques.

Synthesis of gluconic acid have been observed to be influenced more by cations than anions.The effect of various metal ions, viz.,, copper, iron, zinc, manganese, calcium and magnesium on the yield of gluconic acid were studied. The results were compared with a defined medium. The yield of gluconic acid was influenced more by a combination of metal ions rather than individual ions. Potassium ferrocyanide treatment gave the most promising results compared to other treatment techniques. The comparision is mentioned in Tables 14 and 15 (9).

The effect of protease on the synthesis of chitinase by *T. harzianum* is being studied. The inhibition of protease is done by adding the serine protease inhibitor, phenyl methane sulphonyl fluoride (PMSF) at different time intervals in an effort to optimize the time factor for the maximum production of chitinase in media with varying contents of carbon and nitrogen. The time of maximum production was found to be different in each case, the concentration of inhibitor being the same.There is an enhanced production of chitinase in the presence of protease inhibitor and it is observed that there is a lag in the

Table 14. Optimum level of metal ions in various media.

Medium	Optimum concentration (mg/g dwm*)					
	Cu^{2+}	Fe^{2+}	Zn^{2+}	Mn^{2+}	Ca^{2+}	Mg^{2+}
UT	0.0110	0.2551	0.0150	0.0152	12.7260	14.9782
KF	0.0378	0.1721	0.0283	0.0162	5.444	4.4774
SA	0.0387	0.2953	0.0488	0.0326	7.1321	6.0216
TCP	0.0258	0.2894	0.0430	0.0270	7.2482	5.9342
TCPH	0.0330	0.2988	0.0469	0.0401	6.5856	5.5242

*Dry weight of molasses.

Note: The optimum concentration of metal ions was taken as the values above which no significant increase in the yield of gluconic acid was observed.

Table 15. Yield of gluconic acid using various media at respective optimum metal ion concentration.

Medium	Yield of gluconic acid (Yp/s)					
	Cu^{2+}	Fe^{2+}	Zn^{2+}	Mn^{2+}	Ca^{2+}	Mg^{2+}
Defined	0.2746	0.1915	0.3121	0.3980	0.2617	0.3841
UT	0.2333	0.2417	0.2300	0.2439	0.1796	0.2135
KF	0.2784	0.2752	0.2768	0.2857	0.2523	0.2834
SA	0.2287	0.1195	0.2218	------	0.2139	0.2383
TCP	0.2287	0.1982	0.2218	0.2469	0.1604	0.2013
TCPH	0.1921	0.1944	0.2218	0.2421	0.1184	0.1930

UT, Untreated molasses; KF, Potassium ferrocyanide treatment; SA, Sulfuric acid treatment; TCP, Tricalcium phosphate treatment; TCPH, Tricalcium phosphate with hydrochloric acid treatment.

production with the time of maximum production being different (Srividya and Panda, unpublished results).

The physical parameters in reactors such as aeration rate, agitation rate and pH, affecting production of β-1,3-glucanase by *T. harzianum* were optimized using a self-directing optimization technique. A maximum β-1,3-glucanase production of 0.910 U was obtained at a pH (controlled) level of 4.9, aeration rate of 0.91 l/(l)(min) and agitator speed 220 rpm (14). Similar studies were carried out for production of chitinase by *T. harzianum* (Arthur and Panda, unpublished results).

A low cost chloroform shock technique for the release of periplasmic penicillin amidase by *E. coli* cells was optimized using multiparameter optimization technique.The study was also extended to *E. coli* cells grown under different concentrations of phenylacetic acid, glucose and lactic acid. However, the percentage release of penicillin amidase by chloroform shock was more than that of osmotic shock in all the experiments carried out with various levels of glucose and lactic acid separately plus optimal level of phenylacetic acid. Penicillin amidase extracted by $CHCl_3$ treatment is better than the osmotic shock irrespective of the cells grown on different carbon sources (2).

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