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Strategies for improving fermentation medium performance: a review

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Many techniques are available in the fermentation medium designer's toolbox (borrowing, component swapping, biological mimicry, one-at-a-time, statistical and mathematical techniques—experimental design and optimization, artificial neural networks, fuzzy logic, genetic algorithms, continuous fermentation, pulsed batch and stoichiometric analysis). Each technique has advantages and disadvantages, and situations where they are best applied. No one 'magic bullet' technique exists for all situations. However, considerable advantage can be gained by logical application of the techniques, combined with good experimental design.

Keywords: medium design; medium optimization; fermentation; gamma-linolenic acid; neural networks; fuzzy logic; genetic algorithms

Introduction

When developing an industrial fermentation, designing a fermentation medium is of critical importance because medium composition can significantly affect product concentration, yield and volumetric productivity. For commodity products, medium cost can substantially affect overall process economics. Medium composition can also affect the ease and cost of downstream product separation, for example in the separation of protein products from a medium containing protein.

There are many challenges associated with medium design. Designing the medium is a laborious, expensive, open-ended, often time-consuming process involving many experiments. In industry, it often needs to be conducted frequently because new mutants and strains are continuously being introduced. Many constraints operate during the design process, and industrial scale must be kept in mind when designing the medium. Some of these constraints and challenges are summarized in Table 1.

In Michael Crichton's fiction book, *The Andromeda Strain* [36], 'The Wildfire project employed almost every known growth medium', totaling 80 in all. If only this were true! A medium design campaign can involve testing hundreds of different media. One of the more difficult aspects of the medium design process is dealing with this flow of data. In reality, often the information generated from design experiments is difficult to assess because of its sheer volume. Beyond about 20 experiments with five variables it is very difficult for a researcher to maintain medium component trends mentally, especially when more than one variable changes at a time. Data capture and data mining techniques are crucial in this situation.

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Two different improvement strategies: open and closed

Most of the studies published about medium improvement start with the objective of 'given these components of the medium what is the best combination possible?'. This can be referred to as a 'closed strategy' in that it defines a fixed number of components and the type of components used. This is the simplest situation. The disadvantage of this strategy is that many different possible components, which are not considered, could be beneficial in the medium. It considers an extremely limited subset of design possibilities. It assumes you have chosen the right components to start with. The obverse situation, the 'open strategy' asks, 'What is the best combination of all possible components available?'. This situation is much more complex and difficult to deal with. Experimental design strategies do not handle this situation easily. The advantage of the open strategy is that it makes no assumptions of which components are best. The ideal design strategy would be to start with an open strategy, and then move to a closed strategy once the best components have been selected. Too often researchers progress too quickly to a closed strategy.

Three issues are particularly important to consider before medium design starts: the effect of medium design on strain selection; how well will shake flask medium design data scale up; and what is the target variable for improvement.

The effect of medium design on strain development

Medium design is intrinsically linked to strain development and the two processes form a 'Catch-22' circle (you can't choose the best strain until you have the best medium, and you can't design the best medium until you have the best strain). This is because no one medium works best for all the strains being tested. Therefore the question arises, which medium should be used to choose strains. Two options are available. The first is to use the best medium composition based on the results with one strain and then

Table 1 Constraints and challenges that may operate during the process of designing an industrial medium

Encountered in laboratory	Encountered on an industrial scale
• Development time	• Availability of raw materials throughout the year
• Cost of development efforts	• Transport costs of medium components
• Lack of shaker space	• Batch to batch variability of complex medium components
• Precipitation reactions	• Medium cost and price fluctuations of medium components
• Water quality	• Stability of the supply company
• Dispersion or dissolution of solid components	• Bulk storage and handling of medium components
• Effect of components on assay techniques	• Pest problems
• Effect of components on downstream product purification	• Effect of components on broth viscosity or power consumption
• Foaming	• Disposal costs of spent medium
	• Dust hazards

choose the best strain based on this medium. The second option is to choose the strain based on one general medium and then optimize the medium for the best strain. With both these methods there is no guarantee that one of the discarded poor performing strains would not surpass the chosen best strain if a different medium was used.

Instead of doing one-at-a-time development (medium then strain, or strain then medium), considerable benefit can be gained by conducting medium design and strain development simultaneously. An example is given in the study of gamma linolenic acid (GLA) production by *Mucor hiemalis* IRL51 [69]. GLA is an omega-6 fatty acid, which is a component of triglycerides within oil accumulated within the fungi. The oil content of the cell and GLA content of the oil are linked on a maximum GLA productivity curve (Figure 1). By using this generic relation, new strains can be screened on three media (A, B and C in Figure 1) that locate microbial performance on different parts of this curve, giving a picture of strain performance over a wide range of conditions.

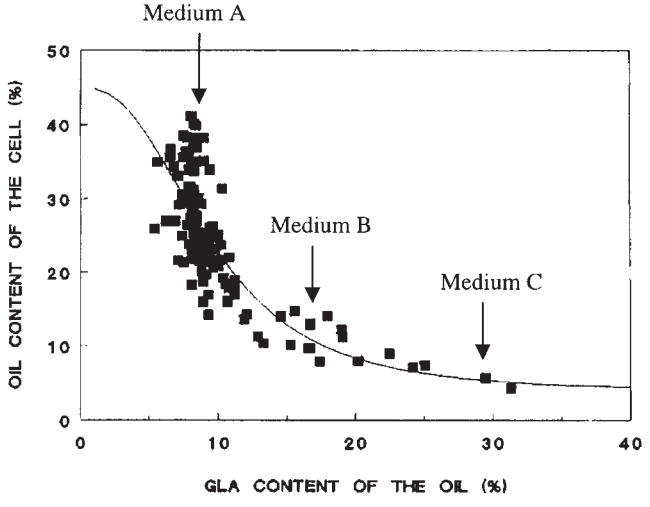

Figure 1 The maximum GLA productivity curve for *Mucor hiemalis* IRL51. Individual points correspond to tested strains and different media. The expected performance of new strains on media can be located along the curve, demonstrating that three media (A, B and C) are sufficient to gauge microbial performance in screening trials. Data are from Kennedy *et al* [69].

The scalability of shake flask results

No matter which medium improvement strategy is chosen, a large number of experiments are usually involved. This large number of experiments necessitates shake flasks, as it is not practical to do large numbers of experiments in stirred controlled vessels. Shake flask systems suffer from at least four weaknesses [70]: the pH is not controlled during the fermentation; the oxygen transfer capabilities of shake flasks is poor; considerable evaporation can take place during shake flask culture; and shake flasks can lack adequate mixing.

Many researchers assume that the best medium chosen from shake flask data will be the best medium in a large-scale stirred tank. The reality is that few rigorous comparisons of medium performance at different scales have been published, and often, on scale-up, the medium composition is changed to take advantage of control strategies, eg the fed batch addition of substrates. For gamma linolenic acid production one scale-up study [70] showed that, using the same medium, biological performance in 10-L fermenters is usually the same as that in shake flask culture (Figure 2). There were some inconsistencies, which could be attributed to scale, but no large, systematic differences were apparent. Considering the immense amount of data reported on shake flask systems, this is comforting, but does it hold true for all systems?

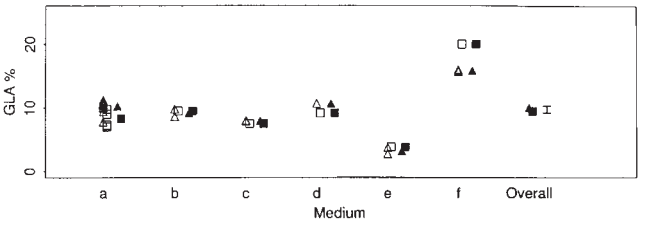

Figure 2 A comparison of medium performance in shake flasks (SF) with that in stirred, pH-controlled 10-L vessels. The performance characteristic is GLA content of the oil produced by *M. hiemalis* IRL51. Symbols show individual results and means for each of six media (a–f), and, as a summary, the overall mean. These results validate the scalability of shake flask results, at least for the system studied. Data are from Kennedy *et al* [70]. △ Shake flask; □ 10-L vessels; ▲ SF mean; ■ 10-L mean.

Target variable

Some medium design studies flounder because the target variable to be improved is not clearly defined. In the production of GLA any of the following performance indicators could be chosen for improvement: GLA content of the oil (%); GLA content of the cell (%); GLA concentration in the fermenter (g GLA L^{-1}); triacylglycerol content of the oil (%); specific productivity of GLA (g GLA g $cell^{-1}$ h^{-1}); volumetric productivity of GLA (g GLA L^{-1} h^{-1}); cost of nutrients/unit GLA (\$ g GLA^{-1}); cost of nutrients/unit productivity (\$ L h^{-1} g GLA^{-1}).

Any of these criteria may be used. For example if the microbial oil is viewed as a competitor for evening primrose oil (EPO), which contains 10% GLA, then a higher GLA content of the oil may be desired, eg 15%. If separation of the GLA from the oil to produce purified GLA is required, then as high as possible GLA content may significantly reduce purification costs. If GLA is thought of as a commodity good then volumetric productivity may be the important variable. It is essential to choose the right target before beginning the design process.

Lexography of medium design

A word of caution should be expressed about the use of the terms 'optimize', 'optimization' or 'optimum'. In the mathematical definition optimization means 'the maximizing or minimizing of a given function possibly subject to some type of constraints' [81]. This implies an objective function that is the target to maximize or minimize, and a possible set of constraints. Under this definition, it is impossible to claim to have the optimum medium, as it is always possible that another, as yet unknown, medium could out-perform the so-called 'optimum medium'. However in the literature many researchers use the term loosely to mean 'the best medium they have come up with to date'. A better way to describe the medium would be to call it an improved medium, or a medium with enhanced performance.

Improvement strategies and procedures

Literature search ('borrow someone else's medium')

Often the first step is to look and see what media others have used to grow the same genus, species or strain. Several handbooks have been devoted to microbial media [8,9]. The problem with this approach is that usually there are too many options, and too much effort is required to test them all. Experience becomes a key factor in assessing published media. For example many published media are laboratory media that can be discarded as an industrial option because they contain a number of expensive components. Sorting out the published media to come up with a shortlist is essential.

Some chemically defined media contain a large number of components eg chromatium medium contains 34 components. Some antibiotic media contain five carbon sources. Some media contain unusual components (Table 2) which are usually related to the substrate the microbe was isolated from, and emphasize a lack of identification of the nutrient requirements of the organism. Some published media suffer from component overloading, which can lead to interactions, precipitations or toxic levels of different components. The 'magic component' can exist in some media. This is a situation where, for no apparent reason, one component seems to perform much better than other equivalent components. Usually these components are complex, and can even be specific brands of the same component, for example corn steep liquor. These logic-defying components argue for an extensive 'open strategy' prior to focussing on the closed strategy.

Table 2 Some unusual microbial media components in the published literature

Blood products (from sheep, horse, guinea pig)	Lima beans
	Eggs
Rabbit dung	Prunes
Commercial rabbit food	Leaf litter
Quaker oats	Hay infusion
Calf brain infusion	Lard
Carrots and tomatoes	Guar gum
Bile	Cocoa shell

Component swapping ('try everything strategy')

One strategem is to take one medium composition and swap one component for a new one at the same incorporation level. This strategy is often used to compare components of one type, eg to compare many different carbon or nitrogen sources [53,104,112]. It is an open strategy and has the advantage that it enables large numbers of components to be compared. It is one of the few open strategies available. Component swapping, however, performs poorly as a total improvement technique because it does not consider component concentrations or interaction effects. It can be best thought of as a screening tool, not so much to find the best medium but to discard the poor performing medium components. It is however, a powerful and useful technique for assessing and understanding microbial regulation. Much of our understanding of carbon, nitrogen, and phosphorus source regulation has been built up from careful consideration of such experiments [27,28,87].

Biological mimicry ('match and win strategy')

The biological mimicry strategy is based on the concept that the cell will grow well in a medium that contains everything it needs in the right proportions. It is simply a mass balance strategy. The composition of the cell, the concentration of cell mass, cell growth yields, and the desired extracellular product concentrations are used to calculate how much of the various components should be in the medium [41,43]. It can be performed on two levels, an elemental level, eg balancing the carbon or nitrogen levels, or a molecular level, eg balancing amino acid or phosphate levels. This approach is popular with more complex cell types, or even for developing whole insect diets where the medium should mimic the cell composition of the host or the insect itself. When no medium has worked to date, or there is no obvious starting point for design, this strategy can have a place. Conducting the mass balance is something that, in itself, is useful because it enables the theoreti-

cal limiting nutrient to be identified and changed if desired. Utilizing yield data also enables a maximum theoretical final cell concentration to be predicted. In essence, the mass balance is a useful check to determine if the medium is in the right 'ball park' in terms of nutrient levels.

The mass balance methodology has some significant limitations. Firstly a detailed elemental composition of the cell is required (Table 3). The elemental composition of a cell can vary quite significantly depending on whether the organism to be grown is a yeast, bacterium or filamentous fungus, the stage of growth, if the culture sporulates, or sometimes simply at the species level. The published data usually apply to common organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Klebsiella aerogenes* or *Pseudomonas* sp. If the organism under test is different from the species from which the published data were obtained, then you are entering uncharted waters using these data.

One solution is to grow some of the desired cells and conduct an elemental analysis. While this overcomes problems with uncertainty in cell composition, cell yield data must still be measured. Measuring cell yield on many different elements is expensive. laborious and time consuming. Such detail is not usually gone through unless the data will have a significant impact and the project is long-term.

Next, the elemental composition of the nutrients must be determined. With pure compounds, this is simply a matter of calculation, but with complex, poorly characterized medium components, eg fishmeal or cotton seed meal, the data can be hard to come by. On top of this, the elemental composition of complex medium components can vary widely from batch to batch, or on a seasonal basis, eg if a different fish species is used to make fishmeal. If the balance is done on the molecular level, eg, balancing amino acid levels, this technique can lead to nutrient over- or under-loading as cells construct or consume cell components. Lastly, balancing the elements does not guarantee success. A well-balanced medium can still perform badly. Simply balancing the medium elemental composition does not address the cell's regulatory machinery, which can dictate substrate preferences.

Table 3 Elemental composition of microorganisms, and growth yields related to macro- and microelements from Ertola *et al* [43]. Such data are useful in mass balance calculations for estimating nutrient levels and a maximum theoretical cell concentration

Element	Elemental composition of microorganisms	Growth yield (g dry biomass g element^{-1})
C	44–53[a,b,c]	1.1[d]
N	10–14[a]	8.75[d]
	7–10[b,c]	9.09[e]
P	2.0–3.0[a]	39.1[d]
	0.8–2.6[b]	
	0.4–4.5[c]	27.7[c]
S	0.2–1.0[a]	333[d]
	0.01–0.24[b]	
	0.1–0.5[c]	
K	1.0–4.5[a,b]	59.5[d]
	0.2–2.5[c]	161.3[e]
Mg	0.1–1.2[a,b,c]	430[d]
		128[e]
Ca	0.01–1.1[a,b]	3.3×10^{3} [d]
	0.1–1.4[c]	8.3×10^{2} [e]
Fe	7×10^{-3}–0.9[a,b,c]	6.7×10^{3} [d]
		1.7×10^{3} [e]
Zn	8×10^{-3}–2.4×10^{-2} [a,b]	2×10^{4} [d]
		2.7×10^{5} [e]
Mn	7×10^{-4}–4.8×10^{-2} [a,b]	2×10^{4} [d]
		7.7×10^{5} [e]

[a]Bacteria; [b]yeasts; [c]filamentous fungi; [d]assumed values for *Klebsiella aerogenes*; [e]calculated values for a *Pseudomonas* sp as determined by continuous culture.

One at a time ('keep it simple')

The rationale behind the one-at-a-time strategy is to keep the concentration of all medium components constant except one. The concentration of this medium component is then changed over a desired range. This strategy has the advantage that it is simple and easy. Most significantly, the individual effects of medium components can be seen on a graph, without the need to revert to statistical analysis. The technique has some major flaws; interactions between components are ignored, the optimum can be missed completely, and it involves a relatively large number of experiments. Because of its ease and convenience, one-at-a-time has historically been one of the most popular choices for improving medium composition [3,47,61,111,113,120,128].

Experimental design ('maths and stats')

Fisher [45] developed the basic theory of experimental design which shows that changing more than one factor at a time can be more efficient than changing only one factor at a time. Applications to medium improvement date from the 1970s and many studies claim substantial improvements over media obtained using 'one-at-a-time'. For example, Silveria *et al* [130] compared 'one-at-a-time' and experimental design for optimizing the medium composition for *Methanosarcina barkeri* growing on methanol. The gas production rate ($CH_4 + CO_2$) could be increased over 1.3 times the rate for the 'one-at-a-time' optimized medium.

To improve a medium using the experimental design approach requires both a design and an optimization technique. The design specifies the medium variants to test in the experiment, including the number of replicates and the arrangement of tests into homogeneous 'blocks'. Then, using the experimental data, the optimization technique employs a mathematical model to predict an improved medium composition.

Designs

Full factorial (complete factorial): In a full factorial design every combination of factor levels is tested. Typical factors are strain, medium, medium component, temperature, initial pH and inoculum. Full factorials provide 'coverage' at the cost of a large number of runs (ie tests or variants). There is a shorthand for full factorials which gives an idea of the number of runs required in an unreplicated experiment: two-factor designs are denoted by $a \times b$ and three-factor designs by $a \times b \times c$. In $a \times b$, the first factor is tested at a levels and the second at b levels; in $a \times b \times c$, a third factor is tested at c levels. This notation

extends to a^n—a complete factorial for n factors, each at a levels. For example, Thiel *et al* [146] experimented with two factors: seven *strains* on eight *media*, using a 7×8 design (56 runs per replicate). Prapulla *et al* [119] experimented with three factors: *carbon* (cane molasses at 4%, 6%, 8%, 10% and 12%), *nitrogen* (NH_4NO_3 at 0, 0.13, 0.26, 0.39, 0.52 g L^{-1}) and *inoculum* (at 10% and 20%) using a $5 \times 5 \times 2$ design (50 runs per replicate). The most commonly used full factorials in medium improvement experiments are two-level factorials (denoted by 2^n when there are n factors). These designs are the smallest capable of providing detailed information on factor interactions (ie antagonistic or synergistic effects).

Partial factorial (incomplete or fractional factorial): A partial factorial design provides a compromise when the number of runs required in a full factorial is impracticable. In medium improvement applications, these are usually two-level fractional factorial designs, but there are some interesting exceptions. For example, Silveira *et al* [130] experimented with 11 medium components, each at three levels using only 27 runs—only a tiny fraction of the 177 147 required by the full factorial, 3^{11}.

Two-level fractional factorials are denoted by 2^{n-k}, where n is the number factors and $1/2^k$ is the fraction of the complete factorial used. This notation gives an immediate idea of the number or runs required. For example, 2^{5-1} is a half fraction of the complete factorial, 2^5, and requires 16 (ie 2^4) runs per replicate. Although some partial factorials provide virtually no information on factor interactions, they are preferable to changing one factor at a time [130].

Plackett and Burman: Introduced by Plackett and Burman [116], these designs are two-level partial factorials most commonly employed for identifying important factors for further investigation. Designs are available for up to 99 factors in 100 runs and the results can be analyzed on a spreadsheet. Theoretically Plackett and Burman designs should be used only when factor effects are additive—when there are no interactions—otherwise the apparent effect of a factor can be enhanced or masked by other factors. In practice, provided the levels of factors are chosen correctly, these designs can produce useful results. Castro *et al* [22] experimented with 20 medium components in only 24 runs, obtaining improvements of up to 45% in gamma interferon production.

Hadamard: A Hadamard matrix is a square matrix used in the construction of partial factorial designs, including Plackett and Burman designs and optimum weighing designs [38]. Applications to optimizing medium compositions and growth conditions were given in White *et al* [161].

Orthogonal arrays (OA): Orthogonal arrays are partial factorial designs which can provide estimates of curvature but not of interaction effects. Theoretically when medium components act additively, orthogonal arrays are optimal for obtaining improved medium compositions. Silveira *et al* [130] described a 'model' application of orthogonal arrays to optimizing medium composition.

Orthogonal Latin rectangles: A Latin rectangle is a rectangular array of symbols, none of which is repeated in any row or column. Latin rectangles can be used to construct partial factorial designs such as the 32-run design for eight factors, each tested at four levels [163]. The resulting designs are known as orthogonal main-effect plans and are most useful when factor effects are additive.

Central composite (CCD): Introduced by Box and Wilson [19], central composite designs are composite designs formed from two-level factorials by the addition of just enough points to estimate curvature and interaction effects. The designs can be viewed as partial factorials with factors at five levels. The number of runs in a central composite design increases exponentially with the number of factors. For two factors, 16 runs are ideal.

Box–Behnken: Introduced by Box and Behnken [17], these three-level partial factorials provide an economical alternative to central composite designs. Like the central composite design, the Box–Behnken design is constructed using two-level factorial designs.

Optimization techniques

Response surface methodology (RSM): Box and Wilson [19] developed a comprehensive methodology employing factorial designs to optimize chemical production processes. The Box–Wilson methodology, now known as response surface methodology (RSM), employs several phases of optimization [18]. The two main optimization phases are: (1) following the path of steepest ascent; and (2) locating a stationary point (using canonical analysis). The key to RSM is representing the yield as a surface. When there are only two medium components, the surface can be visualized as a topographic map in which the height above sea-level is the yield and the medium components provide the grid reference. A path of steepest ascent is a direction in which the surface is rising most rapidly while a stationary point is a point of equilibrium—a summit, saddle or ridge.

Steepest ascent (SA): Steepest ascent requires performing experiments along a path of steepest ascent until the yield falls off. The path is a sequence of variants to the basal medium in which the incremental changes to the medium components are in fixed proportion. Typically a two-level factorial design (either full or partial) is used to estimate the optimum incremental changes required. An application is described in Lieu and Liao [88], where six medium components are optimized using a 2^{6-3} fractional factorial to determine the path of steepest ascent.

Evolutionary operation (EVOP): Evolutionary operation employs factorial designs sequentially to improve yield. The changes made to variables from one cycle to the next are restricted and can only be made when the estimated improvement is greater than the estimated experimental

error. As an example, Banerjee and Bhattacharyya [10] successfully used EVOP to optimize the production of protease by *Rhizopus oryzae*.

Canonical analysis (CA): At some point optimizing along the path of steepest ascent will produce diminishing returns as the slope of the yield surface flattens near a stationary point. Canonical analysis is used to locate and classify the stationary point, either as an optimum, a saddle or some form of ridge [18]. To perform a canonical analysis requires estimating the yield surface using a factorial design such as a full factorial with at least three levels per factor. Chen [25] used a Box–Behnken design to optimize citric acid fermentation by *Aspergillus foetidus*. Haltrich *et al* [55] used a central composite design to optimize xylanase production by *Schizophyllum commune*.

Multiple linear regression: Multiple linear regression, available in most data analysis software, provides an informal alternative to canonical analysis. The first step is to estimate the yield as a quadratic polynomial in the medium components. Then the equation can be differentiated and solved for the stationary point or optimized by a 'derivative-free' method such as the Nelder–Mead Simplex method. To estimate the quadratic polynomial requires a factorial design such as a full factorial with at least three levels per factor [119], eg a central composite [85,103] or a Box–Behnken design.

Gauss–Seidel: Libudzisz *et al* [86] used an experimental version of Gauss–Seidel's iterative method to optimize a four-component medium. When there are *n* components, a single optimization cycle consists of *n* opti-

Table 4 A summary of the designs and optimization techniques used to improve media in 38 published studies. The indicative improvement estimates validate experimental design as a useful improvement strategy

Author [Ref]	Year	*n*	Design	Technique	Software	Estimated improvement
Nair & Panda [103]	1997	3	CCD	SP	Stat-ease	40%
Lee & Chen [85]	1997	2	CCD	SP	SAS	38%
Smith *et al* [134]	1997	4	2^4	ANOVA		
Volchatova *et al* [156]	1997	4	2^4			
Venkat *et al* [149]	1997	3	2^3			
Lao & Schalla [80]	1996	5,8	2^{5-1}, 2^{8-1} [a]		Stat-ease	300%
Jahim & Salihon [59]	1995	5	2^{5-1}, CCD	SP		0%
Liu & Liao [88]	1994	6	2^{6-3}	SA	SAS	22%
Chen [25]	1994	3	Box–Behnken	CA	SAS	
Cruz *et al* [37]	1993	7,6	2^{7-4},2^{6-3}			1100%
Haltrich *et al* [55]	1993	3	2^3, CCD	CA	Statgraphics	330%
Sarra *et al* [125]	1993	2	CCD	SP	SYSTAT	
Venkata *et al* [150]	1993	3	Box–Behnken	SP[a]		236%
Banerjee & Bhattacharyya [10]	1992	3	2^3	EVOP		170%
Castro *et al* [22]	1992	20	Plackett–Burman			45%
Chen *et al* [26]	1992	4	CCD	CA	SAS	60%
Cliquet *et al* [34]	1992	3	CCD		APOLOR	
Prapulla *et al* [119]	1992	3	$5 \times 5 \times 2$	SIMPLEX		
Bloor & England [15]	1991	5,4	2^5, 2^4			
Brückner *et al* [20]	1991	4	2^4			400%
Christen & Raimbault [32]	1991	7	2^{7-4}			
Vigo *et al* [152]	1991	3	CCD[b]	SP[a]		
Silveira *et al* [130]	1991	11	OA			30%
Dodge [39]	1989		CCD			
Houng *et al* [57]	1989	6,5	2^{6-3}, CCD (5)	CA	SAS	60%
Thiel *et al* [146]	1989	2	7×5, 7×8	ANOVA		
Chuiyu *et al* [33]	1988	3	3^3	SP		10%
Libudzisz *et al* [86]	1986	4	Gauss–Seidel			14–23%
Aleksieva *et al* [2]	1985	7,2	Plackett–Burman, 2^2	SA		0% (33%)[f]
Popov *et al* [117]	1985	3	2^3	SA		34%
Salihon *et al* [124]	1983	6	2^{6-1}, CCD	SP[a]		40% (50%)[c]
Rodriguez *et al* [122]	1983	7	Rosenbrock, 2^2			54%
Wright & Richardson [162]	1982	3	3^3	SP		36%[d]
Yakovleva & Bulgakova [163]	1981	8	Latin rectangle			55%
Goldberg *et al* [51]	1980	12				
Bielecka *et al* [14]	1978	4	2^4, CCD			100%
Erdélyi & Kiss [42]	1978	3	2^3	SA		119–130%
Votruba *et al* [157]	1975	5	Rosenbrock			0% (74%)[e]

[a]Inferred.
[b]Modified.
[c]40% yield improvement 50% cost reduction.
[d]36% improvement ruled out by cost.
[e]74% reduction in 4 components.
[f]33% reduction in cost.

mizations in which one component is varied while the others remain fixed. The cycles are repeated until the changes in medium components from one cycle to the next are within a given tolerance.

Modified Rosenbrock: Votruba *et al* [157] and Rodriguez *et al* [122] used an experimental version of Rosenbrock's direct search method for function optimization. Within an optimization cycle, searches for an improved medium variant take place along n directions which are at right angles to each other. Once an improved variant is found, the search directions are updated and a new cycle is begun. The optimization ends when the yield improvement from one cycle to the next is within the limits of experimental error. Typically a direct search method requires $n + 1$ experimental runs in an optimization cycle compared with 2^n in steepest ascent. As shown by Votruba *et al* [157], Rosenbrock's method can determine media with performance equal to media optimized using steepest ascent, but with fewer variants tested.

Nelder–Mead simplex: A simplex is a regular-sided figure in n-dimensions. In two dimensions, a simplex is an equilateral triangle and in three, it is a tetrahedron. The Nelder–Mead simplex method is a 'direct' method for function optimization that does not require derivatives. A function in n variables is evaluated at $n + 1$ vertices (corners) of a simplex, and a direction for improvement is obtained by moving away from the vertex with the smallest value. In an optimization cycle the simplex is updated by replacing the vertex with the smallest value by a new point with a greater value. In an application to maximize lipid production, Prapulla *et al* [119] used the simplex method to optimize quadratic polynomials in three variables. The polynomials were fitted to data using the full factorial 5×5 and multiple linear regression.

Experimental design in action: Table 4 summarizes the design and optimization techniques used to improve media in 38 published studies. Where possible we have tried to give an indication of the yield improvement obtained, either as stated in the text or as inferred by us from the results presented. In all cases the improvement figures should be regarded as indicative only, as they are subject to unquantified experimental error and the possibility of our own misinterpretation.

The designs and optimization techniques summarized in Table 4 are mostly in the tradition of Box and Wilson [19] which includes steepest ascent (SA) and canonical analysis (CA) as components of response surface methodology (RSM). Usually three phases of experimentation can be distinguished: one for identifying important variables ('screening'); and two for optimization. Two-level fractional factorials and Plackett–Burman designs are most commonly employed in phase 1; two-level factorials in phase 2; and central composite (CCD) or Box–Behnken designs in phase 3.

The most common variations on the formal RSM approach are:

(1) 'informal steepest ascent'—pick the best medium as the new centre point or using the signs of the estimated effects with or without regard to experimental error, predict what the best medium might be, using it as the new center point;
(2) analyze a complete factorial using a standard analysis of variance (ANOVA) and comparison of means;
(3) use multiple linear regression to derive a quadratic polynomial model which can be optimized by determining stationary points (SP) or using, for example, the Nelder–Mead simplex method (SIMPLEX).

Three papers [86,122,157] represent a distinct break from the Box–Wilson tradition by employing direct search methods originating in the numerical optimization literature. A direct search design typically employs $n + 1$ experiments in an optimization cycle compared with 2^n and, as shown by Votruba *et al* [157] can determine media with equal performance.

Towards an open strategy

The experimental design method is a closed strategy: the choice of medium components to investigate and their useful ranges of variation must be determined by the experimentalist. However, deciding which components to include and at what levels is probably the critical factor determining the 'success' of an experimental program. This fact is overlooked in many investigations, even when a statistician is involved. The experimentalist will assume that choosing components and levels is part of the statistical design process while the statistician will assume the experimentalist has the prior knowledge to make the correct choice.

Structured preliminary investigations can be helpful, including literature search, analysis of data on the same or related organisms and screening experiments. Screening experiments using partial factorials do form part of the statistical design process, but statisticians would be reluctant to recommend them for many more than ten medium components, severely limiting the scope of preliminary screening. The usefulness of large Plackett and Burman designs in medium improvement is therefore both surprising and encouraging. Additional references include: [24,52,54,74–76,78,83,89–91,95,98–100,118,131,132,137–139,141,144,145,165].

Pulsed injection and continuous fermentation techniques ('a shot in the dark')

The principle behind this group of medium improvement techniques is that in a batch-stirred tank fermenter growth must eventually stop because of depletion of a growth-limiting nutrient. If, when this happens, the correct nutrient is added (often as a pulsed injection) the growth will resume. In practice pulses of nutrients are added until the correct nutrient is identified and growth resumes with the cycle then being repeated. In continuous fermentation the transient increase or decrease in cell density is monitored after pulsed substrate addition. If the substrate is growth-inhibiting, the cell concentration will decrease, until the substrate starts being washed out of the fermenter. If the substrate is growth-limiting, the cell concentration will increase, until the substrate starts being washed out of the fermenter.

The use of these techniques dates back to the chemostat/continuous fermentation research of Mateles and Battat [92] in the 1970s. The system comes with some inherent disadvantages. The first is the time and effort required to set up a stirred fermenter compared to shake flasks. This is especially true for continuous fermentation where up to 10 volume changes may be needed to attain steady state. Experimental progress can therefore be slower than running many different shake flasks simultaneously. For pulsed injection to batch fermentations, eventually a point is reached where toxic microbial products accumulate in the system to such an extent that they become the growth limiting factors. Another disadvantage of the technique is that it improves cell concentration only. The best medium for producing cells is not usually the best medium for producing secondary metabolites. Thus a high cell concentration but low product titer may result. The advantage of the technique is that it is a one-at-a-time approach (although nutrients may be added in groups, eg vitamin or trace nutrient mixtures) and so the exact growth-limiting nutrient at any given instant can be determined. The technique has been used to considerable advantage [50,143,147,164]. A recent development has been the 'carbon source controlled shift technique', with a genetic algorithm applied on-line to design experiments [12]. Although these generic techniques were described almost 25 years ago, their utility has ensured their continued use.

Artificial neural networks ('black box approach')

Artificial neural networks are models that mimic the learning ability of the brain [13]. They take a complete black box approach to modeling data. They are simply 'trained' on a given set of data and then used to predict new data points. This training requires no knowledge or equations to be entered by the user. The network and system remains a black box to the user. Artificial neural networks are not new. They were first developed in the late 1940s, but their popularity and use has increased since the early 1980s as greater computing power became available and inexpensive software products were developed [84]. Artificial neural networks' strong points are that they work well with large amounts of data, they excel at complex pattern recognition, and they require no mechanistic description of the system [109]. This makes artificial neural networks particularly well suited to medium design. Medium design generates large amounts of data that often contain hidden patterns. The artificial neural network is generated by conducting a series of shake flask experiments, and getting the network to learn (to be trained) on this data set. Once trained, the network is given new data points (media compositions) and the output (microbial performance) predicted. Artificial neural networks can perform extremely well at predicting the results of shake flask experiments (Figure 3) thereby saving time and effort.

One study [66] that compared neural networks with full factorial experiments showed that a 63% saving in the number of experiments could be obtained by using neural networks. The accuracy of the technique increases with the amount of data in the training data set but quickly reaches a plateau (Figure 4). Hence a small training set can be sufficient for good predictions on new data.

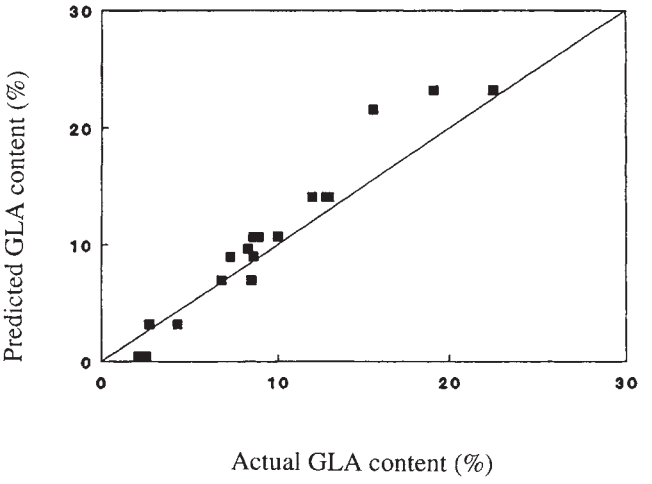

Figure 3 Neural networks can successfully predict the GLA content of oil accumulated by *M. hiemalis* IRL51 in shake flask trials. Individual shake flask results are plotted; ideally, these should fall along the solid line. Data from Kennedy and Reader [68].

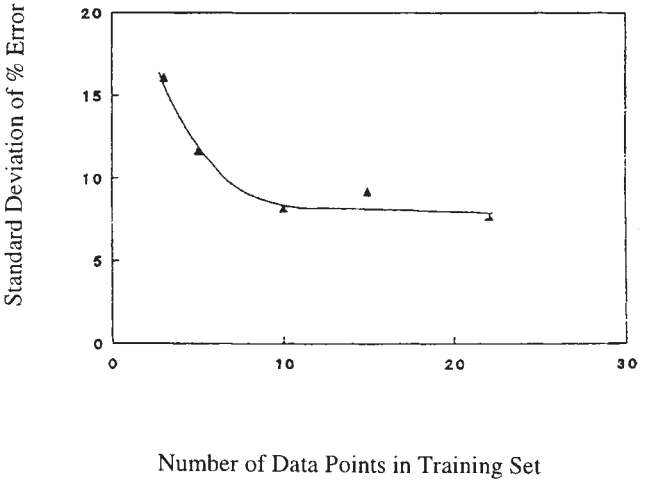

Figure 4 The standard deviation of the percentage error in neural network predictions of biomass in shake flask culture of *Rhodotorula gracilis* CFR-1. The accuracy of the predictions increases with the number of data points in the training set. Data from Kennedy *et al* [66].

Neural networks are, however, not a panacea. They are simply a modeling tool and suffer from all the usual weaknesses of models. For example the quality of data input greatly affects the output, and the greater the amount of extrapolation required the less the accuracy. Neural networks perform best when the data put into them are prefiltered, eg to remove outliers. Neural networks are not tolerant of missing data, eg when one number of an input data sequence is missing. This may occur when cell mass is known, but for some reason product concentration was not measured for the system at the time. Neural networks can also get 'confused' by duplicates, eg if two supposedly identical shake flasks give two very different results. This can cause 'learning difficulties'. There are mathematical 'fixes' to these problems, eg averaging the duplicates and putting them in as one data point, or using a mathematical technique to 'fill in' the missing data. This highlights that

although neural networks are easy to use, some expertise is needed to gain their full potential.

Another problem that highlights this issue is overtraining. This occurs when neural network training is allowed to go on for too long a period. This results in the neural network being extremely accurate at predicting the training data set, but being very poor at predicting a new situation. There is an optimum learning time which can be determined by splitting the training data set in two and monitoring the neural network's ability to predict the second data set.

Neural networks by their ease of use and black box approach can cause the medium developer to lose sight of the microbial system involved. This can be disastrous! An understanding and feeling for how the system performs by the medium developer should be enhanced by neural networks, not replaced by neural networks.

A further development of the technique has been the use of a number of neural networks to attack a number of subproblems instead of using one neural network to predict everything. An example of this is the use of neural networks to predict fermentation time profiles. One neural network is used to determine the phase of the fermentation, ie lag, growth or stationary, and a separate neural network is used to predict fermentation performance depending on the phase determined.

Neural networks can be a good data capture technique and a good training technique for bringing new medium designers up to speed with all the previous data. It allows the user quick access to all the historical data in a form that can be readily used. This is a significant advantage.

Fuzzy logic ('follow the rules')

Fuzzy logic is a multivalued logic which allows membership values (degree of belonging) along the continuum of values between true (1) and false (0). This is in contrast to classical or 'crisp' logic which only recognizes the binary values true and false. A crude example of the difference between classical and fuzzy logic is the classification of a person's height. Using classical logic we may define a person as being tall if that person's height is over 1.8 m. This definition defines a threshold, and consequently a person's height is either clearly within the set 'tall' or clearly outside the set. In contrast, fuzzy logic allows a membership function to be defined with graduated set membership. For example a fuzzy logic definition of tall may expand the previous definition to recognize partial membership for heights between 1.5 and 1.8 m. Partial membership (values between 0 and 1) may indicate that a person is a little tall or is almost tall. Jamshidi *et al* [60] provide an introduction to fuzzy logic for those unfamiliar with the technology [73].

Most importantly fuzzy logic utilizes and executes a series of rules using fuzzy membership functions. The fuzzy membership functions, by defining a degree of belonging, give fuzzy logic the ability to capture and deal with data that do not fit rigorously into present categories. This gives fuzzy logic its key benefit; it deals well with noisy input data in a robust manner.

To use fuzzy logic to design a fermentation medium, the fuzzy membership sets are first defined (Figure 5). This defines to what extent a component concentration of X g L^{-1} will be considered in the low, medium or high concentration sets. Next a set of rules is devised which govern the system (Table 5). These rules are a series of 'if then' statements developed from the results of previous experi-

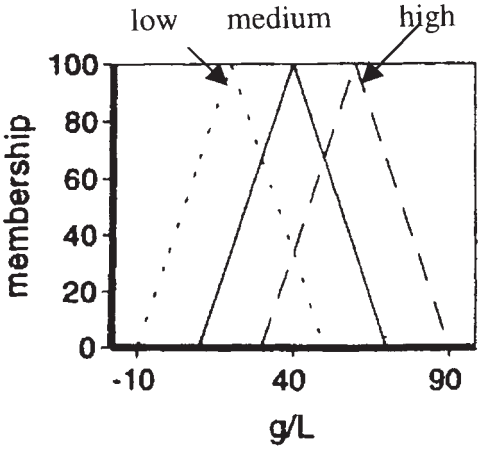

Figure 5 The fuzzy membership set used to define the degree of belonging to each defined category, in this case high, medium or low concentration of the substrate molasses. Data from Kennedy and Spooner [73].

Table 5 An example of a fuzzy logic rule used to design fermentation media. From Kennedy and Spooner [73]. In combination with other rules, microbial performance can be predicted for new medium compositions

If	Molasses is high
	And NH_4NO_3 is medium
	And yeast extract is low
Then	Cell mass is medium
	And lipid content is very high
	And sugar use is very low

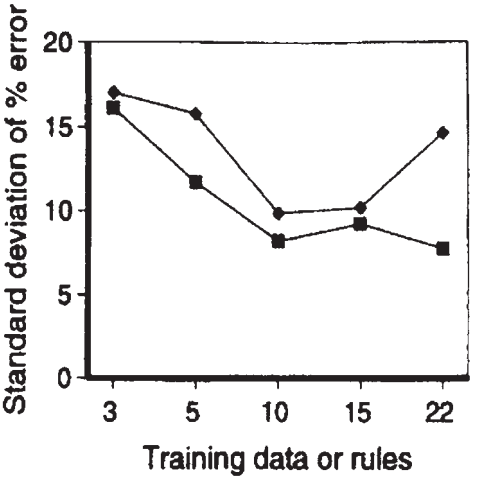

Figure 6 A comparison of the standard deviations of the percentage errors between neural network (–■–) or fuzzy logic (–◆–) model predictions and measured values from a full factorial experiment. The standard deviation is plotted as a function of the number of data points in the training set for the neural networks model, or the number of rules used from the same number of data points for the fuzzy logic model. Neural networks are marginally better than fuzzy rules in predicting cell concentration during the production of microbial oil by *R. gracilis*. Data from Kennedy and Spooner [73].

Table 6 Summary of medium design strategies

Strategy	Concept	Advantages	Disadvantages	Closed or open strategy	Mathematical expertise needed
Borrowing	Obtain medium from literature	• Simple and easy • Good place to start	• May not apply to your strain or situation • often too many to choose from • many are laboratory media with little industrial relevance • publication does not necessarily mean super-performance	Open	Nil
Component swapping	Swap one component for another at the same incorporation level	• not limited to pre-defined components • can try large numbers of components easily • can see regulatory effects of different components	• does not consider effect of component concentration • ignores interactions	Open	Nil
Biological mimicry	Mass balance to determine elemental or molecular composition of the medium	• medium not short of any element • good cross-check of medium composition	• accurate microbial and substrate composition, and yield data needed • complex medium components can vary in composition batch to batch • does not consider regulatory effects	Closed	Low
One-at-a-time	Change the concentration of one component, keeping everything else constant	• simple and easy • easy to grasp results visually on a graph	• interactions ignored • optimum can be missed • large number of experiments	Closed	Nil
Experimental design					
Full factorial	Every possible combination tested	• best possible coverage of experiments	• large number of trials	Closed	Low
Partial factorial	A subset of the full factorial design	• compromise solution, with a smaller number of runs	• only potential average • some high order interactions unobserved	Closed	Moderate
Plackett and Burman	Design for seeing effects of $n-1$ variables in n runs	• the minimum number of runs required • good screening tool • only two levels	• interactions unobserved	Usually closed Open leads to adding multiple similar components at once	Low
Central composite	Partial factorial used to estimate curvature of effects	• estimates curvature of effects not just direction	• moderate number of runs	Closed	Moderate
Box–Behnken	Minimalist central composite	• estimates curvature • low number of runs	• less coverage than central composite	Closed	Moderate
Optimization techniques					
Response surface methodology	Steepest ascent and canonical analysis	• visualize results in 3D	• plotting limited to two variables at a time	Closed	Low if software available
Steepest ascent	Follow steepest trail			Closed	Low if software available
Canonical analysis	Explore around the peak	• distinguishes between peak, saddle or ridge	• only used near a peak	Closed	Low if software available
Multiple linear regression	Polynomial fit to data	• widely used and available	• extrapolation of high order polynomials can produce unusual results	Closed	Low if software available
Gauss–Seidel	Stepping in one direction at a time			Closed	Low if software available

(*Continued on next page*)

Table 6 Continued

Strategy	Concept	Advantages	Disadvantages	Closed or open strategy	Mathematical expertise needed
Modified Rosenbrock	Medium variation along directions at right angles to each other			Closed	Low if software available
Nelder–Mead simplex	Evaluation at vertices of a simplex			Closed	Low if software available
Artificial neural networks	Model that mimics the learning ability of the brain	• handles large amounts of data easily • good at pattern recognition • no mechanistic understanding required	• not tolerant of missing data • can be 'overtrained' • can be inappropriately applied • duplicate results not dealt with well	Closed	Moderate
Fuzzy logic	Multivalued logic which executes a series of rules	• very tolerant of noisy or highly variable input data, and missing data	• needs a set of rules to be devised which can be difficult • rules can conflict with each other	Closed	High
Genetic algorithms	Uses evolutionary natural selection process	• medium steadily improves • new medium automatically selected	• no insight from the designer is used • historical data abandoned on each iteration	Closed	High
Continuous fermentation and pulsed batch	Addition of growth limiting nutrient results in growth	• exact growth-limiting nutrient identified	• effort in setting up a stirred fermentation system • interactions and precipitation reactions can occur if a large number of components are added to a batch system • increased risk of contamination of continuous culture due to the large number of changes to system	Open	Nil
Stoichiometric analysis	Based on intracellular metabolites and pathways	• considers what's happening inside the cell	• considerable simplification (reduction and lumping) occurs	Open	High

ments. When a new medium composition is entered the fuzzy logic program then 'fires' the appropriate rule or rule combination to predict the outcome (microbial performance).

Figure 6 gives an example of predicting cell concentration results using fuzzy logic. As would be expected, as the number of rules increases the accuracy of the model increases. However, as the number of rules continues to increase the accuracy decreases (the standard deviation of the error increases) because some of the rules are conflicting or contradictory. This is analogous to over-training of neural networks. This highlights one of the key disadvantages of fuzzy logic: you have to devise the rules and making up good rules can be a difficult task.

Figure 6 also shows neural networks and fuzzy logic applied to the same data set. Both techniques perform better than conducting a complete set of full factorial experiments, with neural networks performing marginally better on this data set. The strengths and weaknesses of fuzzy logic and neural networks dove-tail together nicely and can be thought of as opposite ends of the spectrum. Neural networks require no understanding of the system, whereas fuzzy logic benefits from an understanding of the system to build rules. A hybrid neuro-fuzzy system could have considerable advantages. Neural networks could be used to design rules, which fuzzy logic could execute in a very robust fashion. To date no one has tried this dual approach to design a fermentation medium.

Genetic algorithms ('evolution in action')

Genetic algorithms mimic the process of mutation and selection fundamental to evolutionary processes and are based on the principle of survival of the fittest. In such algorithms a population of individuals (potential solutions) undergoes a sequence of unary (mutation type) and higher order (crossover type) transformations. These individuals strive for survival: a selection scheme, biased towards fitter individuals, selects the next generation. After some number of generations, the program converges—the best individual hopefully represents the optimum solution [94]. In the case of medium optimization, the use of genetic algorithms can be briefly described as follows. The conditions for each fermentation experiment, ie medium composition, are coded in one 'chromosome', where each medium constituent in the defined concentration represents one 'gene'. After completing the first generation of experiments, chromo-

somes with the highest productivity are selected and replicated proportionally to the productivity. After replication, the crossover of chromosomes and mutation of some randomly chosen genes is performed. In such a way, a new generation of experiments is obtained [168].

The technique can claim some very effective results. In the study of mevinolin biosynthesis by *Aspergillus terreus*, within four generations of fermentation experiments the productivity had increased nearly three times [168]. In the production of formate dehydrogenase by *Candida boidinii*, 14 medium components were optimized within 125 experiments [159,160]. The advantage of genetic algorithms is that they can handle a large number of components and no new guessing is required at each round of experiments, ie the direction is automatically set.

Stoichiometric and metabolic pathway analysis ('inside the cell')

Most medium design techniques treat the cell as a black box or utilize solely empirical data. The next generation of medium design strategies look inside the cell and tailor the medium to the metabolic pathway needs. Stoichiometric models extend the mass balance approach to intracellular components. Current approaches [167] are limited to condensing complicated metabolism into a single equation. Further developments along this theme are expected.

Frequency of use of the different techniques

Table 6 summarizes each of the different medium design strategies and Table 7 shows the classification of the techniques which resulted from a literature search. Some papers used more than one technique and are classified in more than one place if the techniques were used to a significant extent. Table 7 does not reflect total usage of the techniques because the literature search was not all encompassing, and because many studies are conducted in industry and academia that are not published. However, the literature search and Table 7 suggest the frequency of the techniques used and which techniques were emerging. Considering these limitations, the following can be concluded:

- Historically, component swapping or one-at-a-time dominated.
- Since 1980 the use of experimental design has become more common. Possibly it has become more difficult to publish medium design studies that solely use the one-at-a-time methodology.
- When using experimental design, full factorial, partial factorial or central composite are the techniques of choice.
- The Plackett and Burman design has been somewhat overlooked and is a useful screening tool.
- Once the designed experiment has been completed, the most popular methods of analyzing and modeling the data are multiple regression (polynomials), response surface methodology and steepest ascent.
- Response surface methodology has recently emerged because of more widespread access to software packages capable of using the methodology.
- Continuous fermentation (CF) technique grew out of the CF research boom of the early 1970s. It still remains a powerful tool with potential for further developments.
- The clear recent trend has been use of the 'intelligent techniques' namely neural networks, fuzzy logic and genetic algorithms. The wide distribution of inexpensive software will enhance the use of these techniques.

Table 7 Frequency of use from a study of 94 techniques used to design fermentation media obtained from a literature search

Technique	Number of references using the technique
Mass balance [43]	1
Component swapping [53,104,112]	3
One-at-a-time [3,4,11,16,23,29,30,31,40,44,46,47,56,58,79,82,110, 111,113,120,127,135,151]	23
Experimental design	
• full factorial [15,20,33,42,117,119,122,124,134,146,149,156,157]	13
• partial factorial [21,26,32,37,57,59,80,88]	8
• central composite [26,34,39,55,85,103,125,152]	8
• Plackett and Burman [2,22]	2
• orthogonal arrays/Latin rectangles [130,160]	2
• Box–Behnken [125,150]	2
Optimization techniques	
• steepest ascent [15,57,88,124]	4
• multiple regression/response surface methodology [55,85,119,123,153,162]	6
• EVOP [10]	1
• Box–Wilson [2,20,42,117]	4
• Modified Rosenbrock [122,157]	2
• Gauss–Seidel [86]	1
• Simplex [119]	1
Continuous fermentation [12,50,92,101,143,147,164]	7
Neural networks [66,67]	2
Fuzzy logic [73]	1
Genetic algorithms [159,160,168]	3
Total number of usage of all techniques	**94**

When to stop designing the fermentation medium

Designing a fermentation medium can be a never-ending task. When is enough, enough? Sooner or later the medium developer is confronted with the following questions:

- How quickly can I improve microbial performance using medium design? This question is often precipitated by a manager entering the picture and saying 'how long before we have a decent medium?'
- Which method of design leads to the fastest improvement rate?
- How well is my current design strategy working?
- When should I change direction, eg test new components (revisit the open strategy) or concentration regimes?
- When has medium design reached the point of diminishing returns and when should it be abandoned?

Often the decision to stop is arbitrary, eg when the product titer is X. However, a simple curve can go a long way toward answering these questions in a logical fashion. This curve is the plot of microbial performance *vs* number of media tested (Figure 7).

Kennedy [64] demonstrated that these 'medium development curves' showed a characteristic exponential character

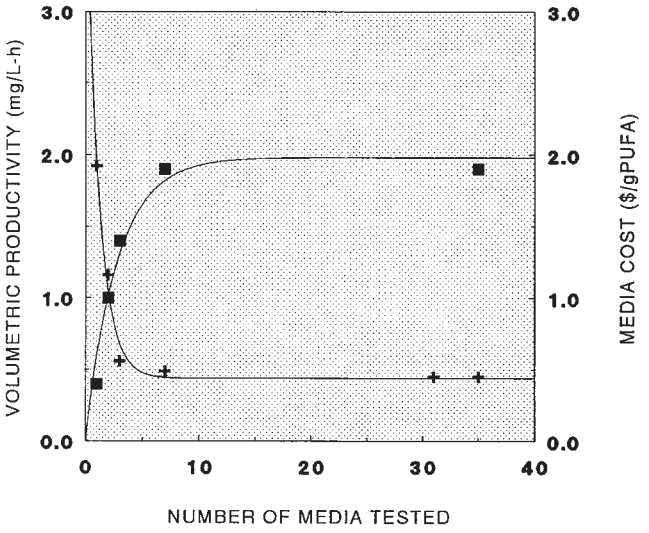

Figure 7 Medium development curve for the production of GLA by *M. circinelloides* IRL 172 (■, productivity data; +, cost data). Once the curve reaches a plateau it's time to review the current design strategy. Data from Kennedy [64].

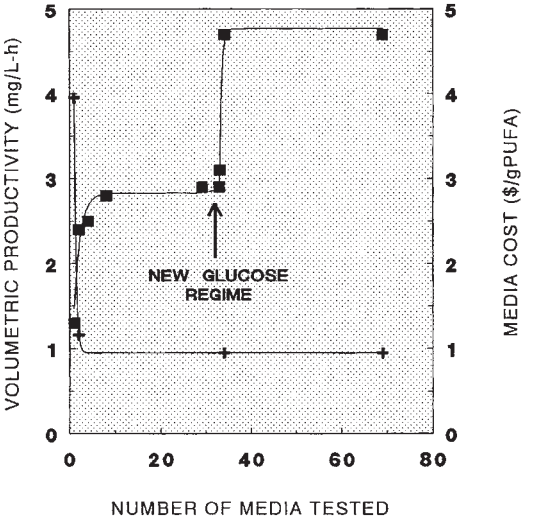

Figure 8 Medium development curve for the production of GLA by *M. hiemalis* IRL 51 (■, productivity data; +, cost data). A plateau in productivity prompted a radical change in glucose regime—with dramatic results. Data from Kennedy [64].

and could be modeled using either of the following equations. For maximizing performance, eg volumetric productivity:

$$Y = A(1 - e^{-BX})$$

where Y = performance indicator, X = number of media tested and A,B = constants.

For minimizing performance, eg medium cost:

$$Z = Ce^{-DX} + E$$

where Z = performance indicator, X = number of media tested and C,D,E = constants.

These curves show (in practice for our laboratory conditions) that often only a small number of media (less than 20) need to be tested before a plateau is reached. When this plateau emerges it is time to either do something radically different or stop. Figure 8 illustrates this point. A plateau was reached after 20 media had been tested. At this point glucose concentrations were changed from 0–40 g L^{-1} to 0–250 g L^{-1} with dramatic results. Significantly, increased productivity, achieved by an increase in medium cost, resulted in no drop in medium cost per unit PUFA produced. Plotting the medium development curve and knowing the kinetic parameters for medium development of your laboratory can avoid a large amount of wasted effort.

An industrial fermentation substrate database

The knowledge base from which fermentation medium design progresses is a description of the substrates that can be used. This most conveniently takes the form of a composition and cost database of substrates available. The database can contain hundreds of industrial products, many of which are agro-industrial byproducts. With a high value fermentation product the fermentation company can have the luxury of using defined pure components. For commodity fermentation products the fermentation company has little choice but to use another industry's waste stream. The other industry is invariably keen to get their waste off site as soon as possible, and to find different uses for the waste apart from the ubiquitous low-value animal feed component.

Table 8 The top five carbon sources available in New Zealand. Adapted from [67,71,121]

Carbon source	Composition assumption	%C	$ kg^{-1}	$ kg^{-1} carbon
Whey permeate	5.1% lactose	2.1	0.003	0.12
Tallow	100% C18	76.0	0.39	0.51
Glucose syrup	67% solids	26.8	0.56	2.09
Methanol	Pure	37.5	0.93	2.49
Raw sugar	100% sucrose	42.1	1.27	3.02

$, New Zealand dollars, 1991 (NZ$ = 0.6 US$).

At a minimum the database should contain an elemental breakdown of the substrate, its cost and if possible its molecular constituents. Elemental sources, eg carbon sources can then be grouped together for comparison. If cost comparisons are needed it is important not to compare the substrates on a $ kg^{-1} basis, but on a $ kg^{-1} of element, eg $ kg^{-1} of carbon, nitrogen etc. Databases of carbon and nitrogen sources available in New Zealand can be seen in Tables 8 and 9 which also illustrate that each fermentation

Table 9 The top five nitrogen sources available in New Zealand. Adapted from [67,71,121]

Nitrogen source	% N	$ kg^{-1}	$ kg^{-1} nitrogen
Brewers grain	2.72	0.01	0.51
Whole whey	0.14	0.003	1.74
Fishmeal	9.6	0.18	1.85
Urea	47.0	2.35	5.00
Slink meal	9.6	0.55	5.73

$, New Zealand dollars, 1991 (NZ$ = 0.6 US$).

Table 10 Effects of different samples of calcium carbonate on penicillin G production. Variability can have a significant impact on biological outcome. Data from Corbett [35]

Brand	Comparative titer (%)
Sturcal	100
Omya D5	62.9
Omya vedar	53.4
Omya calibrite	71.1
Millicarb	57.9
BP brade	70.0

company's database may differ because of regional geographic differences.

Just because a substrate is inexpensive does not mean it is automatically the best. For example, the cheapest carbon sources in New Zealand are whey and tallow. Whey is available only on a seasonal basis in New Zealand and foams significantly in aerated fermenters. To use whey the microorganism must be able to ferment lactose. To use tallow the microorganism must be able to degrade lipid. Tallow is a solid at most fermentation temperatures and some means must be found to distribute the tallow finely and uniformly to enable efficient utilization. Tallow also floats, resisting attempts to break it up using agitation. Methanol is an inexpensive carbon source, which no doubt led to its popularity for single cell protein (SCP) production [77,97,142,158]. In the mid 1980s New Zealand had a gas-to-methanol production plant at Waitara producing 1400 tons per day of methanol [5,6]. However toxicity to most microbes (and to humans) at relatively low concentration levels, and a low flash point (11°C [62]), means methanol is not always desirable [63]. Molasses, long thought of as a low cost fermentation substrate, is not so low cost in New Zealand, which does not have a sugar cane industry and has high transport costs.

The cheapest nitrogen sources in New Zealand are brewers grain, whey, fishmeal and urea. Urea is relatively inexpensive in New Zealand because New Zealand has an ammonia-urea plant. Urea, however, has the disadvantage that water solutions degrade on heating, giving off ammonia [93]; a distinct disadvantage if heat is used to sterilize the medium. Whey is an inexpensive byproduct of

Table 11 The effect of a disc material on production of streptomycin. Leachates can reduce biological performance. Data from Sikyta [129]

Disc surface coating	Streptomycin production (%)
Glass	100
Aluminium	100
Stainless steel	28
Brass	18
Zinc	0
Brass-alloy	16
Copper coating	15
Tin plating	60
Phosphatisation	47
Rubberisation	45
Polyamide resin	109

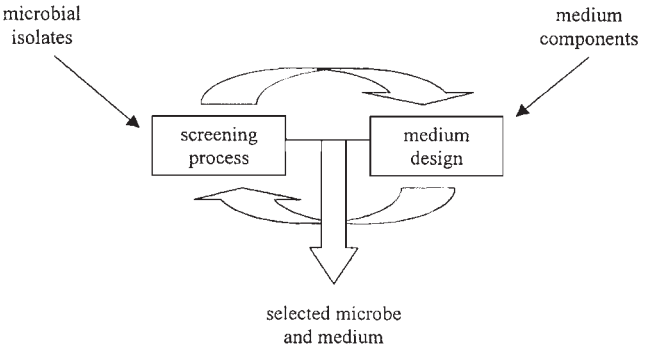

Figure 9 A co-ordinated medium design and microbe screening process has synergistic advantages as well as decreasing the chance of missing a better performing system.

New Zealand's extensive dairy industry. Fishmeal, although inexpensive, may not be used in New Zealand fermentation media because it has a high mercury content by world standards for fishmeal (0.02–2.40 mg kg^{-1} fish [148]). Of particular note are the by-products of the fruit processing industries eg apple pomace, kiwifruit pomace, grape, tomato and citrus processing waste. Prices for these materials are negotiable because they usually have few alternative uses and local councils are increasing pressure to decrease landfilling by increasing disposal costs. Unfortunately these substrates contain high molecular weight polysaccharides and fiber which most industrial microorganisms cannot readily utilize. Usually they are only an option for solid substrate fermentations where they may perform extremely well, eg apple pomace has been successfully tested as a mushroom substrate in New Zealand [65]. Another disadvantage for these types of substrate is transport costs. Usually it is only economic to consider these substrates if the fermentation facility is near the source of the byproduct, as drying and transport costs are prohibitive.

Usually the lowest cost fermentation substrates come with strings attached, ie they are a solid or have some other disadvantage. Therefore price alone should not be the sole selection factor. Tables 8 and 9 illustrate the large financial incentives there can be for considering more-difficult-to-use substrates.

Another question that is frequently raised is where to get composition data for the substrate database. Three sources of data are useful: the classic Solomons [136]; Miller and Churchill [96]; and Zabriskie *et al* [166]. Another good source of data is animal feed composition collections [1,7,49,102,105–108,133,140,153–155].

Tricks of the trade ('traps for young players')

Even when the above elegant and sophisticated approaches to medium design are followed, things can still go wrong. A few of these from the author's experience are recounted for interest.

Complex medium components, eg fishmeal or yeast extract, may vary widely in composition between batches as is often anticipated. Foetal bovine serum (FBS), used in tissue culture, is a well known example. However, when dealing with 'pure' components this variability is assumed not to exist, and so, usually, different brands of pure chemicals are not biologically compared. This can be a mistake.

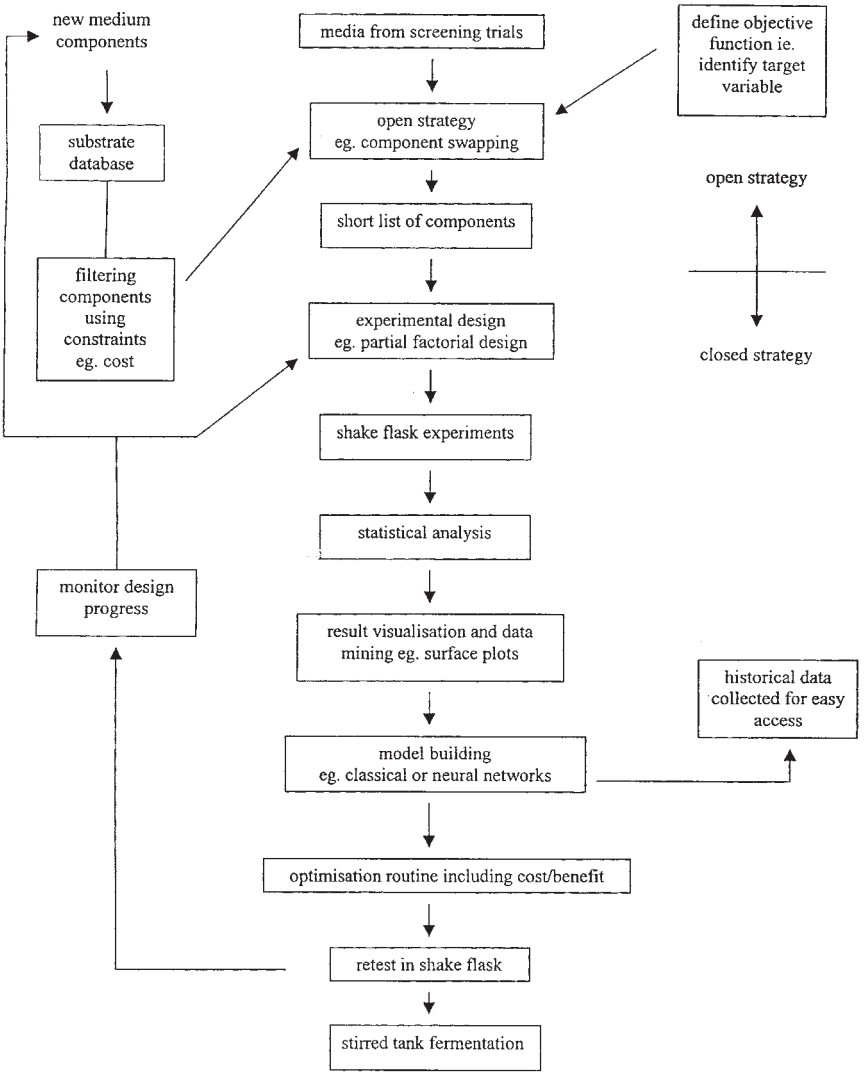

Figure 10 An integrated medium design process.

Table 10 shows that different brands of calcium carbonate had a huge effect on penicillin G production. In this case, the purity and production processes can have a significant impact on biological outcome. This emphasizes the need for a total quality management system with a documented substrate supply.

When designing a medium it is very easy to add a variety of components together. Just because they can be added to water doesn't mean they are available to the microbe. Precipitation can occur upon adding the components or browning reactions can occur during sterilization. One of the less obvious of these reactions is the formation of struvite. Struvite is magnesium ammonium phosphate hexahydrate ($MgNH_4PO_2 \cdot 6H_2O$) and can form as hard insoluble particles in fermentation broth when its solubility limit is exceeded (fermentation is not the only biological system to suffer with struvite; it is also present in some urinary and kidney stones [48], waste water treatment systems, after the application of phosphate fertilizers to soils and after the canning of seafoods [126]). Certain microbial species form struvite, production of which is enhanced by agitation [114]. Struvite particles are resistant to shear and centrifuge down with the cells post fermentation. The particles then must be separated from the cells, if the cells are to be used.

The effect of inoculum conditions on shake flask medium design experiments is often overlooked. Whether the cells are added in 'mid-log' state or in 'stationary' phase can have a significant impact on the results. This becomes important when comparing batches of shake flask results. A control medium should be run with each batch to check batch-to-batch variability. Water quality can affect results especially if the water is excessively hard or because of trace mineral levels. It is important to use the same water source in the laboratory as will be used on the large-scale to prevent this problem.

Glass is usually the material used in shake flasks. However metals in large-scale fermenters can leach into the medium, changing biological performance. Table 11 provides a graphic example of this. Ironically, the ubiquitous stainless steel performed relatively badly. The same con-

sideration should be given to materials of the pipes feeding fermenters, eg leaching from copper pipes may poison the medium.

One factor that can confuse shake flask results is nutrient carryover. This effect is most pronounced in chemically defined media. When the inoculum is added to the shake flask, a certain (usually small) amount of nutrient medium is carried along. This carried-over medium may contain trace components which can affect results. The carryover can also be intracellular in that it may be many cell cycles before intracellular reserves of trace components are exhausted. For example, growth factors can be carried over when tissue culture cells are adapted to a new medium. Often three or more subcultures of tissue culture cells are needed before the conclusion that the new medium supports the cells can be made.

Designing the perfect fermentation medium

Given the above array of tools, how should medium design be approached? An integrated medium design process is proposed [72]. The first step is to integrate medium design and microbial screening to assess as wide a range of microbial performance as possible (Figure 9).

Screening with two media, although doubling the effort, can yield better results. Start with an open strategy and ideally persist with this as far as possible. The most logical open strategy is component swapping, although designs such as Plackett and Burman can be used. This open strategy phase implies a fully documented substrate database and likewise chemical shelf. Most experts in medium design are always on the lookout for new components to add to the database (and always badgering suppliers for samples). The open strategy need not be haphazard. The number of components from the database for the open strategy should be cut down by common sense and considering the constraints listed in Table 1.

After the open strategy phase a short list of components should enter the closed strategy phase. Which tools are best? One-at-a-time should be avoided, but the exact choice of design will be determined by the data needed, eg is a small number of runs desired, is it desirable to explore component interactions, or to home in on a peak. Once the runs are complete, analysis, data mining and result visualization should be conducted. Simply picking the best medium neglects the richness of data available. Trends should be teased out of the data. Model building is the next phase. The key seems to be to explore alternatives. Each model type, eg neural networks or genetic algorithms, has different strengths and weaknesses. Monitoring progress is important and can avoid excessive unproductive experimentation. The last phase before scaleup to stirred tank fermenter is retesting the medium to assess the variability of microbial performance on the chosen medium. The steps are summarized in Figure 10.

Designing fermentation media in the future

Some ideas of how media may be designed 50–100 years in the future are offered as speculation:

Detailed metabolic flux analysis: Eventually, if enough detail on the metabolic flux and regulation of components within (and into) the cell is available, this information should be sufficient to design a medium *a priori*.

Change the organism, not the substrate: If means of accelerating mutation and selection (ie evolution) can be found it should be possible to adapt the microbe to the substrate to enhance performance on low cost substrates. This may become the preferred strategy for commodity product manufacture.

Composition gradient experiments: If microbes could be suspended evenly in a non-diffusive medium with a concentration gradient along its axes (medium design chips), the location of where the microbe had grown/performed the best would enable direct reading of the best medium composition.

'Dining out-microbial style': Systems may be able to be designed to allow motile/mobile organisms to make up their own preferred cocktails.

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