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Semiquantitative Process Screening for the Biocatalytic Synthesis of D-Xylulose-5-phosphate

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Abstract:

In this paper we report a method of semiquantitative process screening or route scouting developed to reduce the number of potential process flowsheets for a multistep biocatalytic conversion. Using the dual-enzyme-based synthesis of D-xylulose-5-phosphate as a model, a variety of possible processes was identified. Using data from a limited number of key experiments describing important process attributes, a screening procedure was proposed. Unattractive processes were eliminated early, and the best candidates were put forward as potential options for subsequent development. The method should prove applicable to a range of process synthesis problems.

Introduction

One of the major limitations in the rapid design of new processes is the necessity to evaluate the fewest number of flowsheets from what is potentially a considerable number of alternatives (sometimes known by the term, route scouting). This is particularly critical early in process development when it is hard to justify effort and resource on process design (especially with pharmaceuticals where there is a high attrition rate). Detailed process modeling therefore is not advisable but semiquantitative techniques that eliminate unfavourable options at an early stage are of particular value. For chemical processes, techniques have been used in a systematic way to evaluate process options for some time, including seminal work by Rudd and co-workers[1,2] who classified a range of industrial examples and drew some clear guidelines outlining a paradigm for eliminating unattractive process options. At the heart of this work were two kinds of activity: process synthesis and process analysis.[3−7] This conceptual design method is now established as a powerful tool for the synthesis of chemical processes.[4] In addition to detailed mathematical approaches,[8,9] recently methods have been refined to include minimization of the time to market, which has become a major preoccupation, especially in the pharmaceutical sector.[5,7]

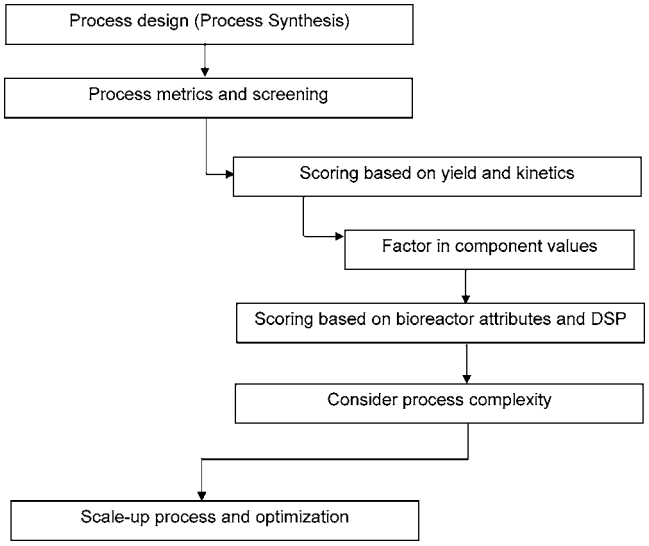

Figure 1. **New paradigm for rapid bioprocess development.**

Many syntheses of complex high-value products currently involve the use of one or more steps of biological catalysis. Biocatalytic synthesis is currently benefiting from advances in genetics, screening, and evolution technologies.[10,11] However, in addition to the scientific knowledge concerning a given biocatalyst, engineering concepts are vital in ensuring effective implementation and development of scalable processes.

A hierarchical process development strategy (Figure 1) for use in biocatalysis can be proposed adapted from those used in chemical engineering. Invariably there are several possible reaction paths to a product, as well as various modes of operation to consider. By gathering basic data on the reaction components and using knowledge gained from a

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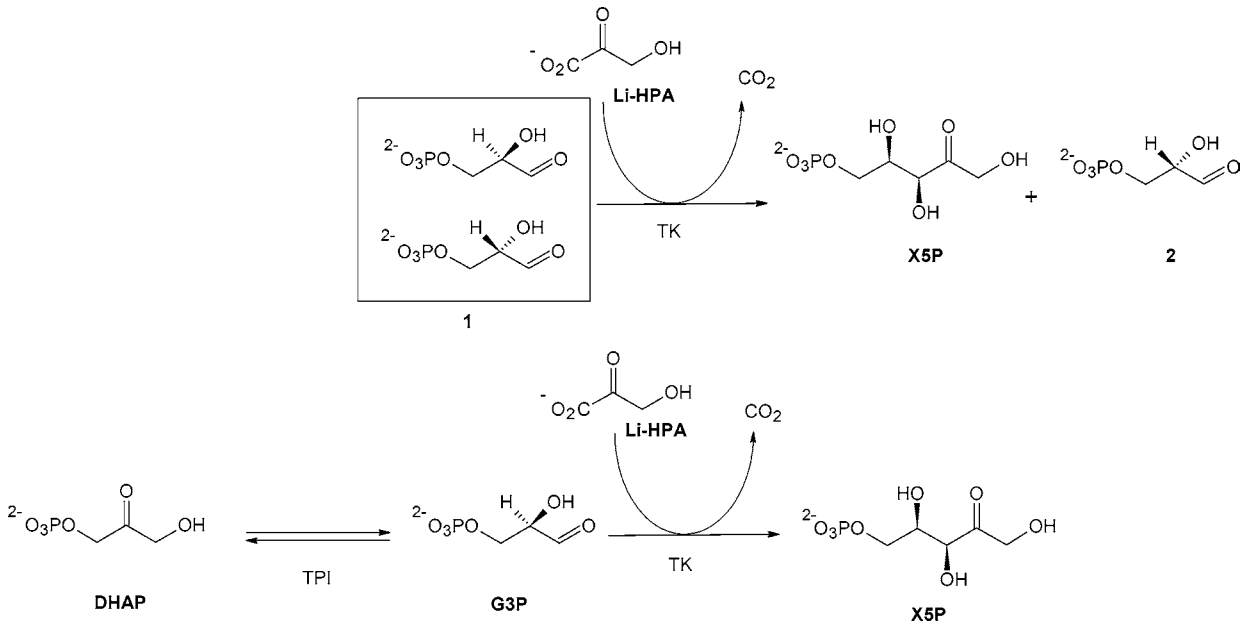

Figure 2. **Enzymatic preparation of D-xylulose-5-phosphate (X5P) either from D,L-glyceraldehyde-3-phosphate (1) with lithium hydroxypyruvate (Li-HPA) and transketolase (TK) to form X5P and L-glyceraldehyde 3-phosphate (2) or from dihydroxyacetone phosphate (DHAP) to form D-Glyceraldehyde 3-phosphate (G3P) using triosephosphate isomerase (TPI) and later X5P with TK and lithium Li-HPA.**

set of key experiments it is possible to conceptualise a variety of process flowsheets.[12,13] Once a set of possible routes or flowsheets are synthesized, it is then necessary to assess their potential viability. Subsequently, a screening criterion is useful to eliminate unattractive processes rapidly. For full route selection, clearly approaches need to encompass SHE (Safety Health Environment), FTO (Freedom To Operate), and robustness issues as well as cost and yield. The procedure we have outlined here deals with cost and yield alone.

The method is particularly applicable to analyzing enzyme cascades or multistep biocatalytic processes[14−16] since a large number of possible flowsheets quickly develop. Collecting data on all the possibilities is not practical in the time scale, and even new methods such as microwell-based parallel experimentation or detailed modeling/simulation will not always provide the information needed to screen a large number of options.[17−19] Hence, we reasoned that with the use of a simple scoring system based on experimental data from a few processes it would be possible to extrapolate (with some assumptions) to obtain estimates for the remaining flowsheets and thus to differentiate between processes and identify the better options or more importantly eliminate the unattractive ones. In this paper we have tested such a method using as an example the multi-enzymatic synthesis of D-xylulose-5-phosphate (X5P)[20] (Figure 2).

Process Screening Methods

Process Options for X5P Synthesis. The synthesis of X5P is a good model to illustrate the methodology of process screening proposed. First, reaction routes were cross-matched with three different modes of operation, namely, batch, fed-batch, and nonintegrated systems (here we define nonintegrated as the separation of the two reaction steps in two reactors). Second, three enzyme combinations are possible: pure transketolase (pTK), triosephosphate isomerase (TPI), and crude transketolase (cTK), which contains trace but significant amounts of TPI. Finally, the synthesis may proceed from DHAP or G3P (see Figure 2). Based on these three sets of options, Table 1 lists the possible process combinations. The important issues that must be considered with each mode of operation are noted in Table 2.

Process Metrics and Screening. Screening here is based on (1) yield and kinetics and (2) bioreactor attributes and downstream processing. The processes were scored on yield of product on substrate ($Y_{[P]/[S]}$) incorporating any equilibrium effects in the system. The kinetics of the processes were described by a score based on the yield of product on enzyme ($Y_{[P]/[E]}$). A bioreactor score was given based on the productivity of the process ($g \cdot L^{-1} \cdot h^{-1}$) and downstream processing (directly influenced by the product stream purity) scores. The

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Table 1. **Process synthesis for X5P preparation listing 20 flowsheets designed with various modes of operation, enzymes, and substrates included**

	mode of operation			enzyme(s)			substrate(s)	
process	B	FB	NI	pTK	cTK	TPI	G3P	DHAP
P1*	•			•			•	
P2		•		•			•	
P3*	•				•		•	
P4		•			•		•	
P5	•			•	•		•	
P6		•		•	•		•	
P7*	•			•		•		•
P8		•		•		•		•
P9			•	•		•		•
P10*	•				•			•
P11		•			•			•
P12	•			•	•			•
P13		•		•	•			•
P14	•			•		•	•	•
P15		•		•		•	•	•
P16			•	•		•	•	•
P17	•				•		•	•
P18		•			•		•	•
P19	•			•	•		•	•
P20		•		•	•		•	•

[a] The table indicates the mode of operation, batch (B), fed-batch (FB), or nonintegrated (NI). The enzymes involved are pure transketolase (pTK), crude transketolase (cTK), or triosephosphate isomerase (TPI). Substrates added to the bioreactor are indicated by glycaraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). Processes with experimental data available (*).

Table 2. **Process synthesis: the important attributes to be considered in different modes of operation**

process	important process issues
batch (b)	- substrate inhibition - substrate value - substrate stability - productivity - thermodynamics - kinetics - impure product stream
fed-batch (FB)	- no substrate inhibition - lower productivity than batch - reaction rate limited by feed rate - clean product stream - equilibrium
nonintegrated (NI)	- multistage process - complex to operate - kinetics or equilibrium

Table 3. **Equations used for calculating process scores for subsequent analysis selection and elimination stages**

process screening score	equation
yield score: product from substrates considering substrate value	$Value_S/([P]/[S])$
kinetics score: product from enzyme considering enzyme value	$Value_E/([P]/[E])$
bioreactor score	$1/(g{\cdot}L^{-1}{\cdot}h^{-1})$
downstream processing score	1/Purity

equations used for calculating the scores are shown in Table 3. Low scores are favourable.

Table 4. **Scores calculated for the 20 X5P processes using component values, yield [P]/[S], yield [P]/[E]**

process[a]	$Y_{[P]/[S]}$	$Y_{[P]/[E]}$	$value_E$	$value_S$	kinetics score	yield score
P1*	0.94	1.61	112	89	70	95
P2	0.98	1.72	112	89	65	91
P3*	0.76	1.75	16	89	9	117
P4	0.61	1.17	16	89	14	147
P5	0.75	1.60	128	89	80	119
P6	0.80	1.50	128	89	85	111
P7*	0.89	2.04	113	13	55	15
P8	0.88	1.10	113	13	103	15
P9	0.40	0.90	113	13	126	33
P10*	0.67	1.50	16	13	11	19
P11	0.86	1.68	16	13	10	15
P12	0.93	1.80	128	13	71	14
P13	1.00	1.23	128	13	104	13
P14	0.66	0.90	113	97	126	148
P15	0.70	0.95	113	97	119	140
P16	0.41	1.00	113	97	113	235
P17	0.58	1.31	16	97	12	168
P18	0.60	1.70	16	97	9	162
P19	0.63	1.43	128	97	90	153
P20	0.80	1.75	128	97	73	121

[a] Actual results are shown for the processes tested in the laboratory (*). For the other untested processes, values and results were extrapolated.

Assigning Value to Reaction Components. The process flowsheets synthesized include different components and modes of operation and will inevitably differ in costs. A correct screening procedure should consider the values of the components as perceived at the time of process development. The value of the starting substrate or enzymes involved is an important factor in determining the viability of a process. For the X5P synthesis model all the components were valued and represented as ratios to give a comparative value (pTK, 112; G3P, 84; cTK, 16; DHAP, 8; HPA, 5; TPI, 1). These values were then factored into the equations for yield and kinetics scores as shown in Table 4. Low scores are favourable.

Calculating Scores for Process Screening. Conventionally data needs to be collected on all possible processes but based on the premise that we could extrapolate, here we have used a set of key data obtained in the laboratory to give approximate process values for the remaining options.[21,22] Table 1 was constructed to make this analysis easier. The experimental data is placed in the table (*), and for the rest of the processes the figures are estimated on the basis of the attributes listed in Table 2. Using a spreadsheet, all the scores for the processes were placed in a matrix to construct a complete table. More specifically, processes P1, P3, P7, and P10 were tested experimentally by using the methods described (see Experimental Section), and the results are shown in Table 4. The productivity and yields of the four processes were used to estimate the attributes of the remaining 16 flowsheets. With the use of the attributes listed

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in Table 2 logical assumptions were made on the yields of the 16 remaining processes. For example P1 is very similar to P2 in terms of the components used, but P2 is run in fed-batch mode. Consequently, P2 should benefit from better yields, since the process is more closely controlled, thus resulting in a more favorable equilibrium and reducing substrate inhibition effects. Therefore, the yields on substrate for P2 (0.98) were estimated slightly higher than those for P1 (0.94). The estimated yields of the unknown processes are importantly based on the best knowledge available and experience. Nevertheless, there will be a degree of error. Such assumptions can be made for processes that are similar to each other with regard to the components involved and mode of operation. This means that the unknown processes with the same substrates or enzymes as the known processes will have estimated yields similar to those of the known processes. The numerical value for these estimated yields is increased relatively based on whether the unknown process is perceived to benefit from a better equilibrium (known process yield + estimated improvement). For unknown processes perceived to suffer from a poor equilibrium, inhibition, instability, or any other constraint, a lower yield is estimated (known process yield − estimated loss). The estimated improvement or loss is a factor that can be small (+0.04 for P2) or great. There is an opportunity here to experiment and estimate different factors in the model as the results become immediately visible. It is important, however, that similar processes with similar attributes such as mode of operation, substrates, or enzymes are estimated with similar yields. Subsequently, the other closely related processes were estimated for their yields and the comprehensive results presented in Table 4.

Results

Screening Based on Yield and Kinetics Scores. Comparing the yield and kinetic scores proved very important in the initial stages of process selection (Figure 3). It is possible to place boundaries on what scores are deemed acceptable and what cost scores are outside the limits. A boundary is placed in the case of X5P synthesis shown by the grey area in Figure 3 to the right of the diagonal line. This boundary is somewhat arbitrarily chosen but in a real industrial case could be calculated on the basis of an economic analysis. A boundary is placed by choosing upper limits to the yield and kinetic scores. By joining the two points together on the plot, a line defines the boundary. For example in Figure 3 the upper limit for the kinetics score is 135. It is possible to move the boundary at a later occasion. The importance of this stage is the possibility of completely ruling out certain processes. Those processes scoring very highly on either axis will not be considered as viable or operable due to inefficiency. The grey area shown in Figure 3 eliminated processes P14−P16 in this case.

Screening Based on Downstream Processing and Bioreactor Scores. In stage 1 of the process selection, a number of possible processes for X5P synthesis were identified, and it was also possible to eliminate a few options (P14−P16). By analyzing them further in terms of downstream (DSP) and bioreactor cost scores, it was possible to further eliminate unfavorable process options. In the grey area in Figure 4 it is shown that processes P4, P9, P11, P18, and P20 in particular seemed to score highly both in terms of bioreactor and DSP cost and are therefore eliminated.

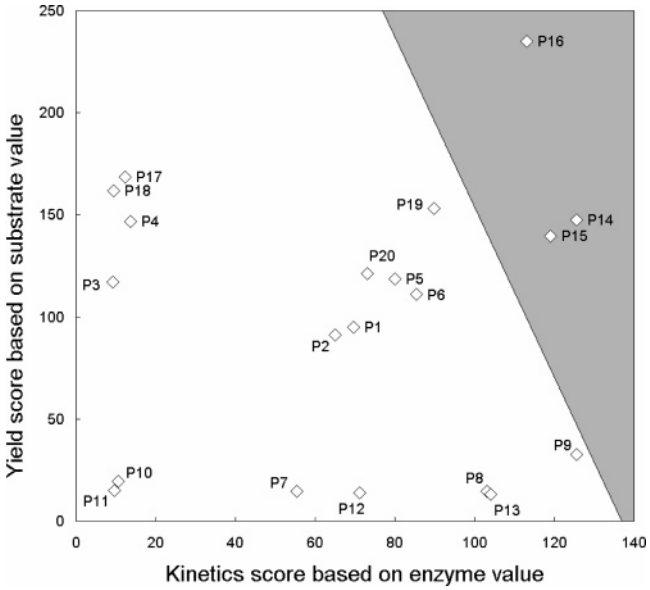

Figure 3. **Stage 1 of process screening (Yield vs Kinetics scores): based on the value of the substrates and enzymes, yield [P]/[S] and yield [P]/[E}. Grey area shows the processes eliminated at this stage based on the boundary.**

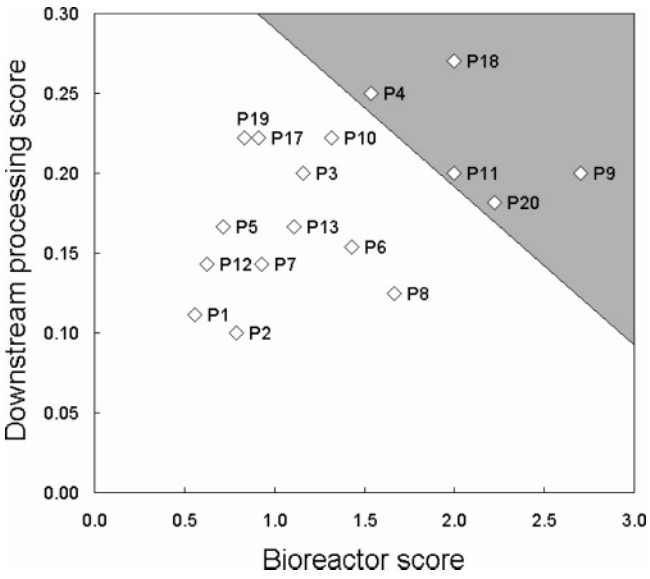

Figure 4. **Stage 2 of process screening (bioreactor scores vs DSP scores): the grey area shows the processes eliminated based on the boundary.**

Process Complexity Considerations. As a decisional tool, a process-screening model must take into account as many factors as possible and not be case specific.[23] In Figure 4 processes P1 and P2 stand very closely in terms of their scores. However, their scores thus far do not indicate how operable the processes are in a given industrial environment. This highlights the need to take into account the complexity of the processes. By this stage of process selection the results

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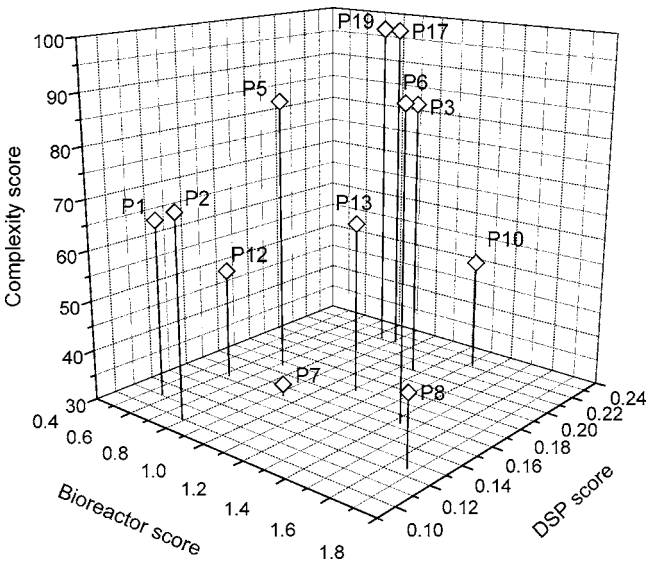

Figure 5. **Considering process complexity together with bioreactor and DSP scores.**

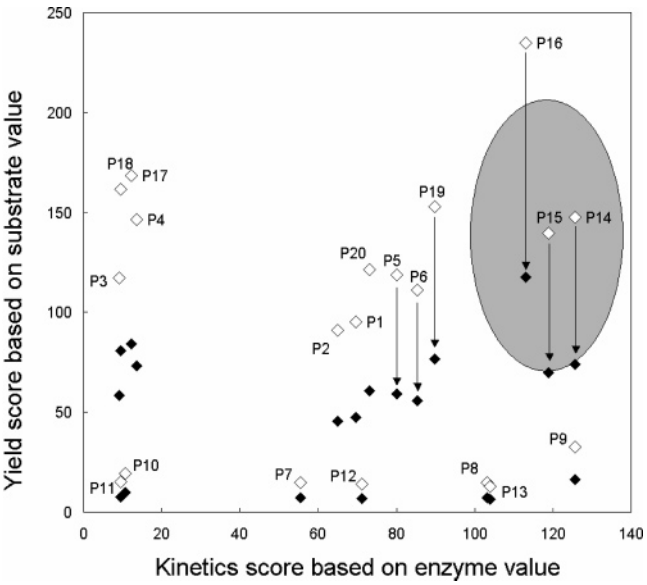

Figure 6. **Sensitivity analysis of the model and where the processes move within the chart as a result of halving substrate values (◆). The grey area indicates the plane of optimization for process 15.**

and the scores had successfully identified the nonviable or poor process options (P4, P9, P11, P14−P16, P18, and P20). The strength of using this system of process selection, we believe, is the early identification of unattractive processes. However, a process that scores well in stages 1 and 2 is not necessarily the easiest to implement in industry. In addition, some of the processes with close scores in Figures 3 and 4 may prove very different in terms of complexity and ability to implement in industry. For example, processes P1 and P2 are similar in their scores, but in fact one is a fed-batch, and the other, a batch system. Hence, we have proposed a "complexity" score based on substrate toxicity, stability, reaction scalability, and byproduct levels. This is a less formal stage of the analysis, and the complexity score has been simply calculated by adding together the numerical scores for the following important factors: substrate inhibition score (15 for G3P and 8 for DHAP), substrate stability score (30 for G3P and 4 for DHAP), byproduct formation score (10 for pTK and 30 for cTK), and scalability score (10 for batch, 30 for fed-batch and nonintegrated). A higher score (these are based on experience) indicates a more complex process to implement. By adding the total complexity score to the model a new plot was used to further distinguish between processes (Figure 5). Consequently, it was possible to eliminate processes P3, P5, P6, P10, P13, P17, and P19. Again, by setting boundaries on the three-dimensional plot for complexity (score must not be higher than 70), DSP and bioreactor score processes were eliminated outside the resulting cube.

Discussion

Methodology. Estimating the values of yields and productivity of the unknown processes is an important issue. The assumption made must be an educated one, based on engineering knowledge. It is possible to assume changes in the equilibrium, yield, and kinetics when the mode of operation changes. In the same way, changes in the reaction components in a process can also affect these values. It is important to note that such screening is only an approximate method of process analysis and the figures assumed should be taken as estimates. The strength of this method is that the values need not be accurate in the initial stages of analysis. Assumptions at this early stage suffice to eliminate a large number of options, which may be impossible to test in the laboratory. At a later stage when more experiments are carried out with more data available, the screening system can be strengthened and improved in terms of accuracy. Experimental values can be easily placed in the spreadsheets, and the results are immediately visible, making this decision-making tool particularly flexible.

Sensitivity Analysis. Given the estimated nature of the scores, the sensitivity of the model was considered important in validating the selection procedure. Sensitivity was analysed by looking at changing given values in the scores. For example, if the substrate value is halved (Figure 6), it is apparent that some processes move across boundaries as a result of a lower-value substrate. This illustrates the sensitivity of the model so that that additional information can be factored and built into the analysis, thus increasing accuracy. Sensitivity analysis of the model also highlights that each process moves within a radius around itself. These planes of optimization can be represented as closed grey planes (e.g., P15, Figure 6) where it is possible to see processes overlapping. This may be a useful analysis in process development where it would show which processes have potential for optimization and which do not. For example, in Figure 6 it is possible to optimize process 15 as indicated by the grey plane. The height of this plane is determined by changes in substrate value or yield, and the width is determined by changes in kinetics or enzyme value. Optimization of each process may result in the process moving within this plane into the accepted boundary, saving it from elimination. The process complexity can also be tested for sensitivity in the same manner. It is possible to change the

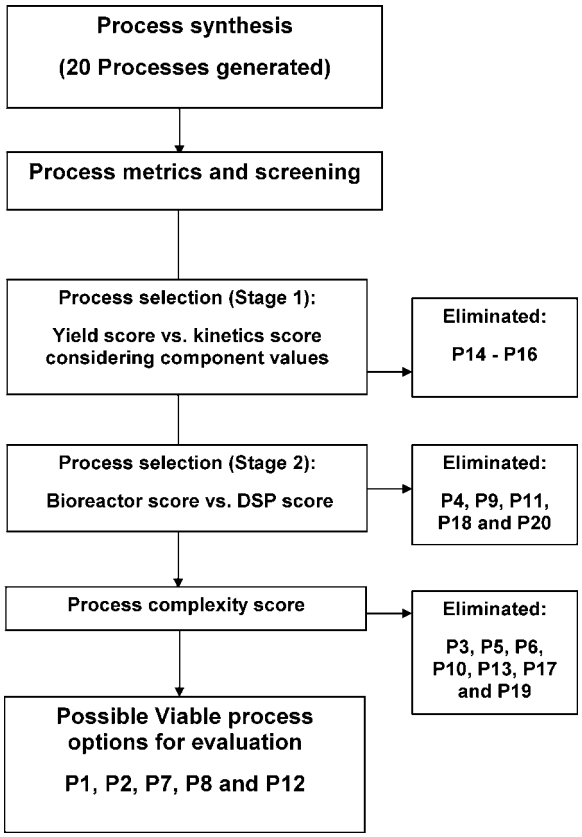

Figure 7. **Overview: Process synthesis, metrics, and screening eliminating unfavorable X5P flowsheets using a logical scoring system.**

weight of one factor, depending on what is more important to the process. For example substrate toxicity, stability, reaction scalability, and byproduct levels were the four factors selected for representing complexity. In the model, factors can be added or removed easily. The weight that factors carry can also be easily changed by simply increasing the score given.

Extending the Model. Scale-up considerations are a logical extension to the existing scores and metrics. On a commercial scale there are often advantages to operating continuously with the materials passing as a continuous stream from one operation to the next. Often the processing is not complete during one pass through the operation, and the recycle of the unreacted materials is necessary. Recycle can cause the build-up of trace materials, and therefore the engineer must give great attention to the impurities of the reaction. Effects that are unimportant in batch operations can overwhelm continuous recycle operations. Likewise, it is possible to take into account utilities in the scoring, such as power, cooling water, steam, gas, acids, and bases as they could become dominant on an industrial scale. The same can be applied to waste. Solvent waste or any liquid waste from a process can seem insignificant in the laboratory-scale experiments but can be critical at larger scales. All these effects can be additional factors possibly built into this screening process and added in the form of simple scores. This will increase the model accuracy and make it a powerful tool for development.

The semiquantitative process screening paradigm proposed here is a possible method to assist in shortening the development time of new biocatalytic routes and processes. From a large list of possibilities it was possible to identify which ones were worthy of investigating further in the laboratory, and unviable options were eliminated (Figure 7). In the work reported here just 4 out of 20 processes were tested in the laboratory. Future work will examine whether a larger or smaller proportion is really needed to optimize the procedure, taking into account the tradeoff between accuracy of the tool and development time. Bioprocesses have rarely had the opportunity to be developed in this way due to the need for a large amount of experimental data. The short-cut method proposed here has potential for improvement and alteration based on the needs of the process engineer.

Experimental Section

All reagents used were of analytical grade and were obtained from Sigma-Aldrich Co Ltd., (Poole, Dorset, UK) with the exceptions of DHAP and TPP, which were donated by Research Specialties, Sigma Aldrich (Buchs, Switzerland).

Four batch processes were carried out in the laboratory (P1, P3, P7, and P10). Processes were run in glass, baffled bioreactors (250 mL), where the reaction mixture contained HPA (100 mM), TPP (2.4 mM), Mg^{2+} (0.9 mM), TPI (20 $U \cdot mL^{-1}$), and mercaptoethanol (10 mM). Depending on the process option, the reactor contained transketolase (1 $U \cdot mL^{-1}$) or either clarified (cTK) source (0.12 $U \cdot mg^{-1}$) or purified (pTK) source (0.5 $U \cdot mg^{-1}$). Processes were initiated by the addition of starting substrate (20 mM G3P or DHAP). Taking samples regularly and using HPLC to analyze for X5P monitored the processes and reaction profiles. Yields based on substrate and enzyme together with productivity ($g \cdot L^{-1} \cdot h^{-1}$) were calculated using the data on the concentration of product.

The mode of the reaction was kept as batch (temperature at 25 °C), and the substrate concentrations were kept below inhibitory levels. The pH was kept at 7.0 using the pH-stat apparatus and 1.0 M HCl. The conversions were monitored for 4 h. The bioreactor was agitated at 150 rpm (the reactions were found to be insensitive to mixing—data not shown). Monitoring and controlling the pH within reaction vessels was carried out using a VIT90 video titrator (Radiometer Ltd., Copenhagen, Denmark).

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