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Investigation of mathematical methods for efficient optimisation of aqueous two-phase extraction

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Abstract

Mathematical strategies were applied to optimise the extraction of recombinant leucine dehydrogenase from *E. coli* homogenates and endoglucanase 1 from culture filtrates of *Trichoderma reesei* in polyethylene glycol–phosphate systems. The goal was to test mathematical tools which could facilitate the optimisation procedure in aqueous two-phase systems. A modified simplex approach, the method of steepest ascent and a genetic algorithm were successfully applied and compared. The methods differ in the height of the optimum found, the number of experiments and the time required. The genetic algorithm proved to be an optimisation procedure which can be used well in aqueous two-phase systems. The simplex procedure has to be further improved.

Keywords: Aqueous two-phase systems; Optimisation; Simplex; Genetic algorithm

1. Introduction

Due to the mild conditions and easy scale up extraction of proteins, aqueous two-phase systems (ATPSs) are an attractive separation technology, especially for the primary product recovery step. A recent review can be found in the Encyclopaedia of Bioprocess Technology [1].

For industrial and laboratory-scale purposes it is important to know if extraction of a desired protein in an ATPS is an option in downstream processing and which parameters lead to an optimal separation and yield.

Johansson et al. [2] stated that there is no clear physical picture about phase separation and partitioning behaviour and Zijlstra et al. [3] mention that one of the reasons why ATPSs are not yet widespread in industry is the lack of adequate mathematical models for prediction.

Due to this lack of theoretical knowledge there is a need to investigate efficient experimental procedures for process optimisation using ATPSs.

In the past mainly heuristic rules were applied for the optimisation [4]. These heuristic rules, however, have some drawbacks, e.g., they are often not accessible for users, who are not familiar with ATPSs.

The main tool for process optimisation in ATPSs currently appears to be the variation of one parameter after the other. Following this procedure the optimum might be missed as demonstrated in Fig. 1. There the experimental points are marked by crosses and circles. Baughman and Liu [5] reported a similar shape for the partition coefficient of lysozyme when plotting the molecular masses of dextran vs. polyethylene glycol (PEG).

A more recent approach for the optimisation using

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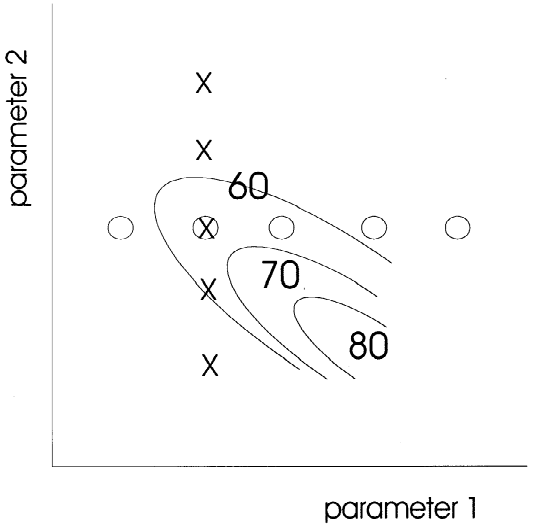

Fig. 1. The possible disadvantage of the variation of one parameter after the other. Crosses and circles are experimental points, the symbols are marking subsequent experimental rows, e.g., first the crosses are measured, the best point is taken for the next series marked by circles. The optimum is missed.

ATPSs is the application of a factorial plan and consequently a second-order regression. This can work perfectly, but since the surface of a space with *n* parameters is often irregularly shaped it may result in a non optimal solution. In addition this procedure is problematic if the optimum is close to the binodial.

Recently several groups investigated the optimisation process for ATPSs. For example, Bompensieri et al. [6] optimised the cloud-point extraction using Triton for maximal lipase activity in the detergent phase, by a regression of second order.

Zijstra et al. [7] optimised the cell partitioning of hybridoma and Chinese Hamster Ovary (CHO) cells by identification of the key factors. The authors performed a regression covering 69 experiments using a 33 point design. In the study, wide variations of single parameters were explored and potassium phosphate was identified as the key factor for the partitioning of cells.

Hart et al. [8] also employed a multifactorial experimental approach, mapping the phase separation while identifying conditions to enrich the target protein, non-native IGF-1, and biomass in opposite aqueous phases. The authors were able to exclude parameters with few experiments as insignificant and therefore could investigate many different parameters, including, e.g., chaotrop concentration and temperature.

In this investigation several mathematical approaches were used for the optimisation of protein extraction in ATPSs, a simplex procedure, the method of steepest ascent and a genetic algorithm.

These algorithms were applied separately for the optimisation of parameters in ATPSs and compared to identify a mathematical procedure which can find a high optimum, possibly the true optimum, within a reasonable time and expense in these complex systems. As an example the normalised yield was chosen as optimisation target.

Mathematical tools were investigated and adapted for the optimisation of PEG–salt extraction. This should be done in a real optimisation procedure.

In principle a mathematical description of the target function could be found and the optimisation methods applied for this function. However a mathematical function can only reflect a part of reality. This investigation will show that it is not possible to derive a suitable mathematical function without mapping all parameters very densely with experimental points. Therefore a real optimisation procedure was investigated.

2. The algorithms

Mathematical handling is difficult for experimental data of extraction in ATPSs. This can be a consequence of rather large errors in the data collections or if discrete solutions are searched, e.g., in the molecular mass of polymers employed.

The algorithms should fulfil the following requirements.

- Sufficiently robust, to override local optima
- Sufficiently detailed, not to miss a potentially small optimum (small with respect to the parameter range)
- Not too sensitive to measurement errors
- No need for distinct values (M_r PEG is only available in a certain variety)
- It should accept that the parameter value used is different from the one the program ordered (ex-

ample: M_r PEG is only available in a certain variety, measurement deviation)
- Interactions with the user should be possible

The choice of suitable algorithms were limited by these demands. The basic features of the three procedures selected are briefly introduced below. All mathematical algorithms tested are used in such a way that starting points are randomly chosen, either by the user (simplex and steepest ascent) or alternatively by the computer program (genetic algorithm). After having performed the first set of experiments the experimental result is fed into the program which calculates the parameters for the next experimental points.

2.1. Simplex algorithm

The basis for this algorithm is the movement of a structure with $n+1$ corners in the space of n parameters.

Nelder and Mead [9] presented the algorithms with the following possible movements: (1) the experimental point with the worst result is mirrored through the middle of all other remaining points of the structure with $n+1$ corners. (2) The experimental point with the worst result is moved to the middle of the other points. (3) The last altered point is moved on the line away from the structure.

We further developed the algorithm with the possibility that (4) the worst point is mirrored at the baseline.

These movements of experimental points are illustrated in Fig. 2.

In addition the distance from the worst data point to the new experimental point is varied for every movement. This is done using a weighted least-square approximation through a polynom of second order of all measurement values in the area.

At the time of the investigation the program could only propose one new experimental point at a time. The next data point was only generated after the previous single experiment was finished.

2.2. Steepest ascent

Steepest ascent (e.g., Box) is a procedure leading the experimentalist closely towards an optimum.

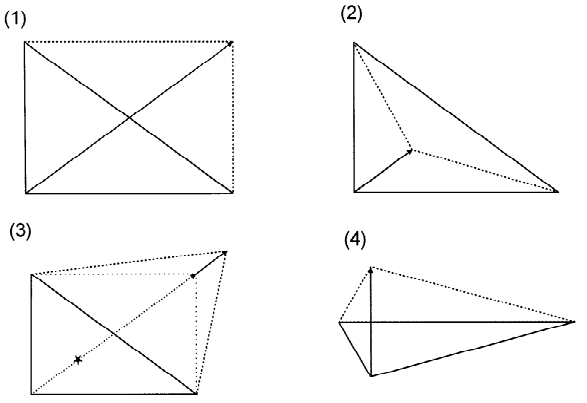

Fig. 2. The possible movements of experimental points using the simplex algorithm. (1) The experimental point with the worst result is mirrored through the middle of all other remaining points of the structure with $n+1$ corners. (2) The experimental point with the worst result is moved to the middle of the other points. (3) The last altered point is moved on the line away from the structure. (4) The worst point is mirrored at the baseline.

Based on the chosen set of parameters and the results of starting experiments a vector is formed by a regression of first order. This vector points towards the direction of steepest ascent. Exploratory runs performed along the path of steepest ascent are indicated by crosses in Fig. 3. The highest value found on the path then forms the base for a new design from which a further advance might be possible. The vector is scaled to allow the construction of distinct measurement points. The steepest ascent can therefore only be a first step in an optimisation procedure. For the ease of operation the steepest ascent was conducted with a partial factorial

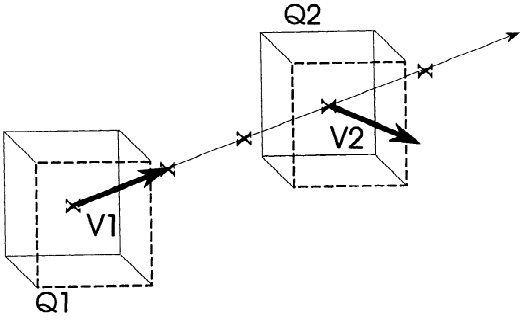

Fig. 3. The method of steepest ascent used in this investigation: Q1 and Q2 are the first and second sets of experiments, V1 and V2 are the vectors pointing to the direction of steepest ascent.

design with 10 experiments per row for four parameters.

2.3. Genetic algorithm

Holland developed the theory of genetic algorithm between 1962 and 1975 [10].

One of the main differences to simplex and steepest ascent is that no model or regression is needed at all. The genetic algorithm differentiates like nature between a genotype and phenotype (see Fig. 4). The parameters selected and the results of the experiments are coded in the program in order to work with more simple structures than the complex ones in reality. In nature the coding is done by transcription and translation while the program ''Galop'' (IBT, Forschungszentrum Jülich, Jülich, Germany) codes the parameters into bit strings. This allows one to perform genetic operations existing in nature, such as selection, crossover and mutation. It is necessary to carry out sets of experiments to generate populations. One population is equivalent to one experimental row. The size of the population and the relative importance of the three operations have to be pre-set in the program.

The number of individuals was set to 10. The crossingrate, which gives the probability that two individuals are exchanging the genes was set to 0.95 and the mutation rate was set to 0.1 leading to a change of one gene in one individual in each population. As a randomiser which is crucial for the genetic algorithm the roulette method was chosen. The best experiment of the last generation was always kept. Other values which have to be set by the user are, for example, the upper and lower boundary for each parameter and the length of the coding.

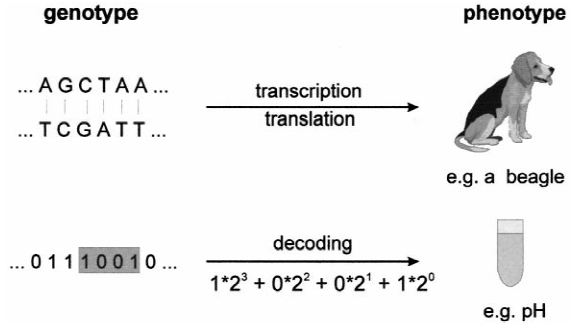

Fig. 4. The principle of nature (top) compared to the genetic algorithm (bottom).

3. Materials and methods

3.1. Chemical reagents

PEGs of molecular mass 200, 300, 400, 600, 800, 1000, 1500, 3000, 4000, 6000, 10 000, 12 000, 20 000, 36 000 were a gift from Clariant (Burgkirchen, Germany) and used as received.

Phosphates and other salts were analytical grade and obtained from Merck (Darmstadt, Germany). Benzonase was also purchased from Merck.

NAD (β-nicotinamidadenindinucloetide) was obtained from Gerbu Biotechnik (Galberg, Germany) and MUC (4-methylumbelliferyl β-D-cellobioside) was purchased from Sigma–Aldrich (Deisenhofen, Germany).

3.2. Strains and fermentation

Leucin dehydrogenase (LeuDH) was expressed in *E. coli* BL21 [pIET98] as described by Ansorge and Kula [11].

After cell separation a 20% suspension was made adding 100 m*M* Kpi buffer of pH 8.0. The cell disruption was performed using a bead mill (LME 0.5, Netzsch, Selb, Germany). The cell homogenate was stored in small portions at −20°C until use.

Endoglucanase 1 (EG1) was produced by *Trichoderma reesei* in a 250-ml shake flask cultivation, according to Mandels and Weber [12].

3.3. Aqueous two-phase extraction

The experiments were conducted in graduated centrifugal tubes. For each experiment 5 ml of probe was used. The pH was adjusted by addition of KOH (addition of acid was not necessary due to the low pH of potassium dihydrogenphosphate). PEG and salts were added and the mass was adjusted to 10.0 g on a table-top balance.

A 100-U amount of benzonase was added to LeuDH-containing samples before mixing in the

overhead shaker to avoid difficulties with the viscosity.

The system was mixed for 2 h in an overhead shaker and subsequently centrifuged at 4000 rpm for 25 min. All experiments were conducted at room temperature (21–24°C).

The volumes of the heavy and light phase were read and a sample of each phase was taken for analysis of enzyme activity.

3.4. Analysis of samples

The enzymatic activity of recombinant LeuDH was determined at 340 nm at 30°C using a Beckman DU5 photometer (Beckman Coulter, Munich, Germany). The standard assay mixture for the oxidative deamination reaction contained 0.1 *M* glycine, 0.1 *M* NaCl, 0.1 *M* NaOH (pH 10.7), 10 m*M* L-leucine and 3.4 m*M* NAD^+. One unit of LeuDH is defined as the conversion of 1 μmol leucin per minute.

The enzymatic activity of EG1 was measured using MUC as substrate. EG1 hydrolyses the β-glycosidic bond and fluorogenic 4-methylumbelliferone is released, which was quantified using a fluorometer equipped with a 360-nm excitation filter and a 455-nm emission filter. Cellobiohydrolase 1 also hydrolyses the substrate and therefore is inhibited by addition of cellobiose (C-7252, Sigma).

EG1-containing liquid is added in an appropriate dilution to a buffer containing 50 m*M* sodium acetate buffer (pH 5), 0.6 m*M* MUC and 4.6 m*M* cellobiose. The mixture is incubated at 50°C. The reaction is stopped after 10 min using 2% Na_2CO_3, pH 10. One unit corresponds to the conversion of 1 μmol methyllumbelliferyl cellobioside per minute.

3.5. Calculations

The normalised yield Y' is defined as

$$Y' = \frac{a_T V_T}{a_0 V_{sample}} \quad (1)$$

where a_T is the activity in the top phase, a_0 is the volumetric activity of the enzyme in the original sample, V_T is the volume of the top phase and V_{sample} the volume of the added supernatant or homogenate, respectively.

The partitioning coefficient K is defined as

$$K = \frac{a_T}{a_B} \quad (2)$$

and the yield Y is defined as

$$Y = \frac{1}{1 + \frac{V_B}{V_T} \cdot \frac{1}{K}} \quad (3)$$

3.6. Software

The improved and adapted version of the simplex procedure was implemented into the program Scilab (Inria, Institut National de Recherche en Informatique et en Automatique, Le Chesnay, France, public domain software).

The regressions for the steepest ascent were performed using the program SAS System for windows, release 6.12 (SAS Institute, Cary, NC, USA). All other calculations were done using Microsoft Excel ‘95 and ‘97.

The computer program ‘‘Galop’’ was used for the genetic algorithm. The program was developed in the Institute of Biotechnology 2 of the Research Centre in Jülich, Jülich, Germany. Originally it was written for the optimisation of fermentation procedures [13].

4. Results and discussion

4.1. What to optimise

If a mathematical procedure should be applied, first it has to be decided which optimum should be found, i.e., which value is the target value to be optimised. This is an important topic even if it sounds trivial. For example optimising the yield is one option, optimising the partition coefficient or selectivity are other possibilities, but lead to different results.

In PEG–salt extraction one would like to have the maximum yield, a purity as high as possible, a high concentration factor, low chemical costs, etc.

However, for the use of optimisation procedures and in particular for a mathematical optimisation procedure it is necessary to select a single value or a

combination of values forming together a single target (e.g., by weighing) which should be optimised.

If an ATPS is used as a clarification step only, the target is the normalised yield of the protein of interest for the systems in which the cells remain in the heavy phase.

Another goal could be to optimise the yield and the purity [note that the equation target $= Y_{target}/Y_{total\ protein}$ is not an adequate tool since, e.g., $1/0.1 = 10$ and $0.1/0.01 = 10$ give identical results, therefore an additive term should be included, e.g., in the denominator leading to $Y_{target}/(Y_{total\ protein} + 100)$].

Optimisation of the apparent K could also be favoured as described by Hart et al. [8]. Economic considerations, physical properties of the phases for subsequent downstream processing steps and many other combinations are possible and applicable to treatment by the algorithms.

The normalised yield Y', defined in Eq. (1) was chosen as an example for this investigation.

Contrary to the yield Y, which is mainly reported in ATPS experiments Y' includes the mass balance. The importance of this choice is demonstrated in Table 1. The used enzyme EG1 seems to have an improved partitioning towards high-molecular-mass PEGs and higher salt concentrations (not shown). Unfortunately, with the increase in these two parameters the protein loses activity. If the partition coefficient or the yield was the target, we would obviously end with an infinite K and a yield Y of 100% despite the fact that the active protein might be recovered only in a few percent.

If cells were present in the light phase or if there was no phase separation obtained the normalised yield Y' was set to $Y' = 0$.

Table 1
The importance of a good choice of the optimisation target is demonstrated for endoglucanase 1 from *Trichoderma reesei* from culture supernatant[a]

	K	Y (%)	Y' (%)
Q1	0.2	9	8
Q1	7.6	75	69
Q1	2.6	65	46
Q1	1.2	43	11
Q1	0.3	12	10
Q1	0.4	13	9
Q1	0.3	19	15
Q1	9.2	86	63
V1	8.0	83	61
V1	35.1	96	48
V1	126.5	99	32
V1	Infinite	100	40

[a] Q1 marks the values obtained in the starting experiments while V1 describes the first vector calculated from Q1.

4.2. Choice of parameters

Several parameters are generally known as important for protein partitioning in PEG–salt systems, these include: the molecular mass of PEG [M_r (PEG)], the amount of PEG [n (PEG)], the choice of salt [SO_4/PO_4/citrate, etc.], the amount of salt [n (salts)], pH, temperature, the kind and amount of biomass, other auxiliary chemicals introduced.

Some of these parameters are less important or cannot be changed in an industrial process. For example the host organism and the expression level was decided upstream in product development and will usually not be changed once a fermentation process is set up.

The choice of salt cannot be included in the algorithms so that one salt, potassium phosphate was selected in advance. The number of parameters was kept small even if the algorithms are in principle not limited by the numbers of parameters handled. The following parameters were used for the investigation: M_r (PEG), n (PEG), n (salt), pH, and for the genetic algorithms in addition the concentration of NaCl was included.

The molecular mass of PEG was always used as its logarithm in the investigation to equal the distances between two molecular masses.

Phosphate was used as phase forming salt in all experiments.

4.3. Results

4.3.1. Steepest ascent

EG1 is a hydrophilic protein and is not expected to partition very favourably in PEG–salt systems to the top phase rich in PEG. The results of a steepest ascent approach are summarised in Table 2. The EG1 partitioning improves with increasing molecular mass of PEG. On the vector the amount of phosphate and the molecular mass of PEG are increased at the

Table 2
The parameters and yields found for the partitioning of LeuDH

	Simplex	Steepest ascent	Genetic algorithm	Lucky experiment
M_r (PEG)	1000	1000	300	1000
n (PEG)	11.7	18.0	21.3	16.0
n (KH_2PO_4)	9.9	16.0	11.9	10.0
pH	7.8	6.0	7.2	6.0
NaCl	–	–	1.6	–
Normalised yield Y'	65	77	80	87

same time. Deactivation occurs under these conditions, however, and the measured values on the vector showed increasing partition coefficients and yields while the normalised yield decreased.

For LeuDH the method of steepest ascent was surprisingly successful. The highest starting value of 44% could be increased to 60% on the first vector and 77% on the new start design. This value could not be reached again in following vectors and designs. Even if the high yield looks like a great success of steepest ascent, we have to remember that steepest ascent cannot work well close to the optimum. The large variations in yield with only minute variations in parameter – (1550; 16.0; 14.7; 5.7) yields 17.5%, (1550; 16.1; 15.3; 5.9) yields 51.9% – do not allow the procedure to actually find the right direction. A high value in the second start design was only good luck, similar to a mapping procedure after the first vector. For LeuDH no regression of second order could be applied to come closer to the optimum due to the vicinity of the phase binodial.

Therefore it was concluded that the method of steepest ascent was best applied for PEG–salt systems at the beginning of an optimisation procedure. In the case of endoglucanase the approach was limited by denaturation of the enzyme. If this happens the method of steepest ascent is not an adequate tool. It may be possible to improve the normalised yield Y' of 57% further by a regression of second order.

For some proteins the response surface of the parameter area is so irregular that it is uncertain if steepest ascent can be applied. The application of steepest ascent is further limited close to the optimum, which is an inherent feature of the method.

Steepest ascent offers a good possibility to show the direction of a high target value. Due to its low demands in programming knowledge it can easily be applied and since only few measurement points are needed it could be used as starting point before turning to other algorithms.

4.3.2. Simplex

The modified simplex algorithm was applied for the optimisation of LeuDH extraction. The highest normalised yield was obtained in experimental row number seven with an normalised yield of $Y' = 65\%$. The experiments were stopped after experimental row number 12. The simplex staggered around with respect to molecular mass of PEG and was tumbling between reasonably high yields and very low ones or no yield (Fig. 5). This observation was due to two facts. It is an inherent feature of the simplex algorithm that it does not choose the most direct way since the new parameter points are generated by mirroring one already investigated experimental point. The more important reason is most probably

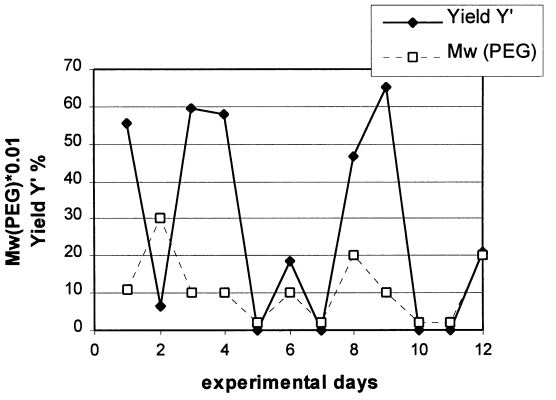

Fig. 5. Experimental results using the simplex algorithm. Yield Y' of LeuDH and the molecular mass of PEG are shown versus the experimental days. One experimental row is defined as 1 day. For the start, simplex, the best experiment, was chosen.

the experiments in which phase separation did not occur. In this cases the yield was defined as zero. The simplex algorithm as used cannot distinguish between ''no yield'' and ''no phase separation''. Having reached a point without phase separation close to the binodial, the simplex will remember that it is not worthwhile searching in this area. Other data points however are indicating that there must be an optimum in the specific direction. And indeed an optimum for the LeuDH extraction is very close to the binodial. The simplex procedure is very slow due to its current design with only one experiment per experimental row. Because of the lengthy procedure described under methods only one experimental row can be performed per day.

However, the investigation indicates that simplex could be very useful for ATPSs if a distinction between ''no phase separation'' and ''no yield'' is introduced into the program and if the experimental rows are enlarged.

Such a modified simplex should be strong close to the optimum through a decreasing structural size. It could be a partner for the steepest ascent.

4.3.3. Genetic algorithm

The genetic algorithm was applied for the optimisation of LeuDH extraction. Since the genetic algorithm can cope with a large number of parameters the concentration of NaCl was introduced as a fifth parameter.

Ten experiments were conducted in a row. The maximum of 80% occurred after five experimental rows (see Fig. 6). The sixth and last row did not give higher values except for some measurement deviation.

The genetic algorithm proved to be a reliable method also for the optimisation of protein extraction in PEG–salt systems. The necessary experimental rows are of reasonable size, the number of experiments, however, is still very high. The genetic algorithm had problems finding a reasonable yield in the beginning. The randomly designed experiment conducted in each row did not lead to an increased yield. This indicates that an adaptation of the installation parameter in the program ''Galop'' could lead to a faster approach to the optimum. As soon as a reasonably high yield of, e.g., 50% is reached the area of allowed values in the computer program

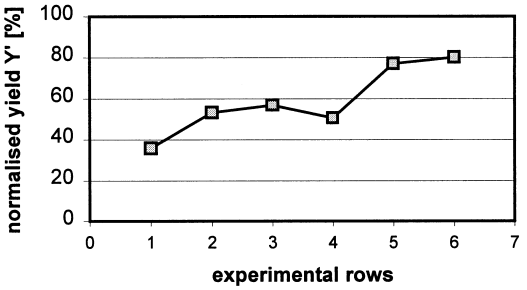

Fig. 6. Experimental rows for the optimisation of the normalised yield of LeuDH using the genetic algorithm. The best result of each experiment is shown. The smaller value in row number 4 compared to row number 3 occurred due to a mistake by the user. The algorithm itself always suggests to include the so far best experiment in the next series.

should be limited since it usually, e.g., does not make sense to search at a pH of 12 if it is known that the optimum can be found towards low pH values. Close to the optimum the option of random mutation is not required any more.

In general the genetic algorithm works for the extraction of proteins in ATPSs. Since it is not based on any assumption it can easily cope with the irregularities as already explained. It is neither harmed by very small values nor is it limited at the maximum. The number of experiments in a generation and the number of experimental series can probably be reduced by better adjustment of the parameters in the program.

4.3.4. Comparison of the algorithms

To indicate the differences in the application of the algorithms, the experimental time for the optimisation vs. the yield is shown in Fig. 7 taking LeuDH as an example. One experimental row is set to 1 day. With respect to time simplex is the least favourable since only one experiment per row is actually possible per day. The genetic algorithm involved one more parameter (NaCl) which did not seem to have a significant influence however. The picture changes if we look at the number of experiments until the maximum was reached. Using the simplex procedure 13 experiments were conducted, 25 for the steepest ascent and 50 for the genetic algorithm. This indicates the potential of steepest ascent if the analytical procedures allow one to carry out more experiments at a time.

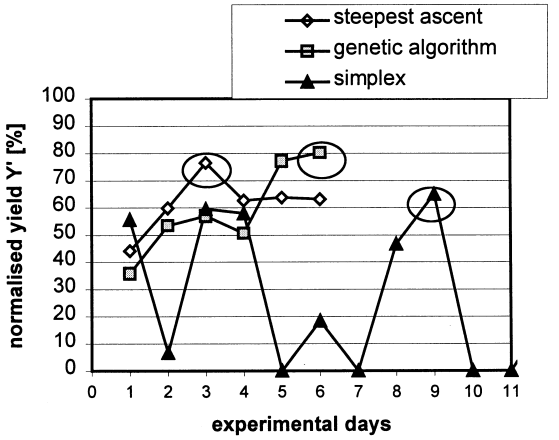

Fig. 7. Experimental time for the optimisation of the normalised yield of LeuDH. For simplicity one row of experiments was set to the time of 1 day. The optimum of each investigation is circled.

The maximum yield found by the different algorithms varies as well, from 65% (simplex) to 76% (steepest ascent) to 80% (genetic algorithm). It has to be pointed out that the very good result in the steepest ascent happened due to good luck as discussed above. The steepest ascent cannot be a tool for an optimisation close to the optimum [14].

By an intensive search close to the optimum an even higher yield of 87% could be found for LeuDH. This maximum was found randomly and nothing indicated this small but distinct maximum. This result should not indicate, however, that a mathematical procedure is not worthwhile since it is not very economical to carry out an optimisation without mathematical tools and hoping that good luck will do it, despite the fact that good luck is not excluded if an algorithm is used.

LeuDH isolation by extraction of *B. cereus* homogenates in PEG–phosphate systems has been described previously [15] and varies significantly from the experiment presented here. This is most probably due to the fact that we used a different host and omitted the heat treatment step which led to a loss in activity in the *E. coli* homogenate. The parameters reported in literature are close to the binodial and did not lead to phase separation in the experiments with *E. coli* homogenates.

It is unrealistic to expect that the global optimum will be found by a few experiments. This is only possible if the response surface is mapped with an infinite small grid. Therefore small local optima are possible as found here by a series of experiments with LeuDH close to areas suggested by the steepest ascent and the genetic algorithm.

Steepest ascent can only be used in the beginning of an optimisation procedure, the simplex algorithm has to be further adapted to compete with the genetic algorithm.

The investigation demonstrates that the heuristic rules used for the optimisation of extraction processes in ATPSs can be replaced by mathematical procedures. The genetic algorithm is a procedure which is ready for use. The algorithms can however be further developed through the knowledge gained in this investigation.

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