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Medium optimization for the production of the aroma compound 2-phenylethanol using a genetic algorithm

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Abstract

Using a genetic algorithm, 13 medium constituents and the temperature were varied to improve the bioconversion of L-phenylalanine (L-phe) to 2-phenylethanol (2-PE) with *Kluyveromyces marxianus* CBS 600. Within four generations plus an additional temperature screening, corresponding to 98 parallel experiments altogether, a maximum 2-PE concentration of 5.6 g/l, equivalent to an increase of 87% compared to the non-optimized medium was obtained. The vitamin content of the medium had to be raised significantly.

Keywords: 2-Phenylethanol; Genetic algorithm; Yeast; Flavor; Fragrance; *Kluyveromyces*

1. Introduction

2-Phenylethanol (2-PE), an aromatic alcohol with a rose-like smell, is the second most used alcohol in perfumery and cosmetics. Currently chemical synthesis is predominantly used for the world's annual production of 7000 t [1]. Rising consumer demands for natural food additives [2] make microbial production of 2-PE, which can be marketed as a 'natural' flavor, economically attractive for food applications. Natural 2-PE yields a price approx. 250 times higher than its chemically produced counterpart.

Numerous food grade yeasts are able to produce 2-PE via the Ehrlich pathway (Fig. 1) when provided with the amino acid L-phenylalanine (L-phe) as a precursor [3]. The process is growth associated but subject to product inhibition by the higher alcohol. Previous experiments showed that the addition of oleyl alcohol as an in situ extractant is suitable for overcoming product inhibition by 2-PE and it increases the aroma production considerably [4].

It was shown that 2-PE production also depends on the medium used. While *Kluyveromyces marxianus* CBS 600 produced 0.21 and 0.26 g/l at 35 °C in a synthetic medium without and with in situ product removal respectively, the strain yielded 0.89 and 3.03 g/l when the glucose in the medium was substituted by sucrose in the form of beet molasses [4]. These findings led to the conclusion that a strategic medium optimization might significantly further improve the product formation.

2. Theory

2.1. Medium components

In addition to a carbon and nitrogen source, $MgSO_4$ and buffering substances the non-optimized medium also contained bacto yeast nitrogen base (YNB) without amino acids and ammonium sulfate (Difco Laboratories, Sparks, USA). This mixture of vitamins and minerals is supposed to supply yeasts with the necessary micronutrients while maintaining the option to use any desired carbon and nitrogen source. Relatively small amounts of YNB were dosed since we initially expected non-limiting growth conditions due to the presence of molasses as a C-source rich in additional nutrients. Indeed, the previous experiments had shown that the carbon source strongly influenced the aroma production. With molasses a much higher 2-PE concentration was obtained than with pure glucose, as this by-product from sugar beet refining does not only deliver sucrose but also a vari-

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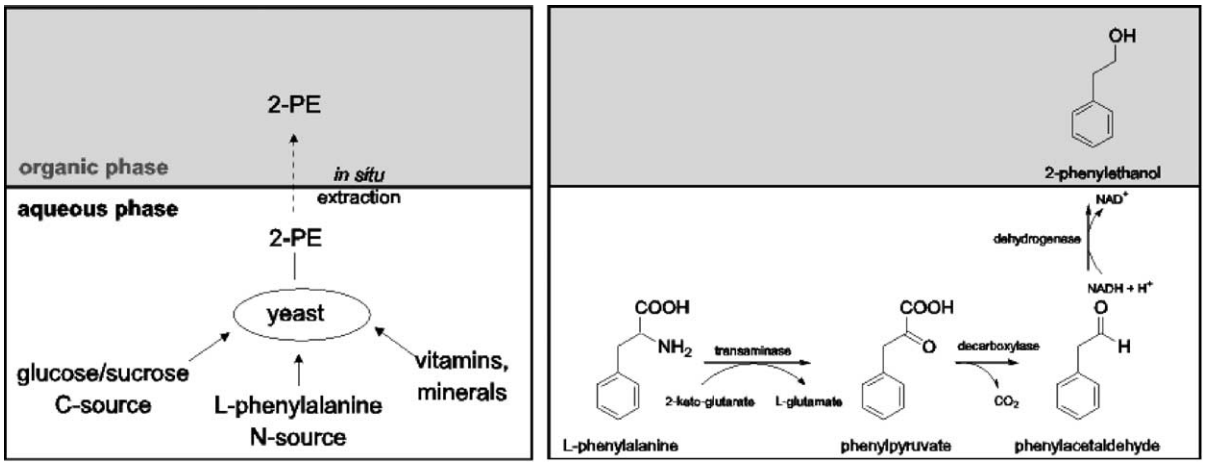

Fig. 1. Ehrlich pathway with in situ product removal.

Table 1
Final concentrations of cations, heavy metals and vitamins depending on their source or literature recommendation

	Aqueous solution with 30g/l sucrose by molasses (mg/l)	Aqueous solution with YNB (10% of recommendation) (mg/l)	Optimal concentrations according to [7] (mg/l)	Optimization required
Cations				
Mg	10	10	49–97	×
Na	680	4	Not necessary	
K	3180	29	78–156	
Ca	160	4	<40	
NH_4	30			
Heavy metals				
As	4.00×10^{-4}			
Al	2.13			
B	0.94	0.013	0.0043	
Cd	0.05			
Co	0.12		0.0059	
Cr	0.22			
Cu	0.12	0.002	0.095	
Fe	4.98	0.009	0.057–0.167	
Hg	0.01			
I		0.080	Not mentioned	×
Mo		0.093	0.14	×
Mn	2.76	0.015	0.11–0.22	
Ni	0.48		0.59–5.28	
Pb	0.23			
Zn	2.90	0.162	0.26–0.52	
Vitamins				
A (retinol)	4.2 IU/l			
B12 (cyanocobalamin)	0.004			
E (alpha-tocopherol)	0.01			
Folic acid		0.00002	Not mentioned	
B2 (riboflavin)	0.02	0.02	Not mentioned	×
B6 (pyridoxin)	0.006	0.04	Not mentioned	×
Biotin		0.00002	Yes	×
B1 (thiamin)	<0.006	0.04	Yes	×
Calcium pantothenate		0.04	Yes	×
Niacin		0.04	Yes	×
Inositol		0.2	Not mentioned	×
p-Aminobenzoic acid		0.02	Not mentioned	×

IU: international units.

ety of minerals and trace elements. Its disadvantage is the high content of metal ions, which can be detrimental to the growth of microorganisms [5], thus an optimal amount has to be found that balances the pros and cons. As molasses is a seasonal product, care was taken to use well homogenized material from the same production batch for the whole set of experiments. The concentrations of cations, heavy metals and vitamins, which arise from adjusting an aqueous solution to a sucrose content of 30 g/l with molasses, are listed in Table 1. The final concentrations of the YNB components used in our previous experiments are also listed in Table 1.

K. marxianus is a well described species in the yeast kingdom [6], however no information about specific nutritional requirements was found. As a rough estimate of the type and amount of nutrients required, the concentrations given by Walker [7] for yeasts in general were used to decide which parameters required optimization (see Table 1).

Because of its fundamental role for the metabolism [8], magnesium was a high priority component in the optimization. All other cations, anions (not shown in Table 1) and heavy metals were present in such high concentrations that an excess was more likely. Exceptions were iodine and molybdene which were not detected at all in the molasses.

As the molasses used was deficient in vitamins, all the vitamins present in YNB were used as optimization parameters except folic acid, which is not mentioned as a special limiting factor for yeast nutrition [7,9] and the number of optimization parameters in the genetic algorithm was to be kept as small as possible.

2.2. Genetic algorithm

The conventional method of optimizing fermentation media is a one-dimensional search with a successive variation of variables. With so many variables however, it is impossible to find an optimum with a realistic number of experiments. Various statistical experimental design methods are known to reduce the number of experiments while scanning a large variable space. Among many possible options stochastic approaches based on genetic algorithms proved to be especially efficient for medium optimizations [10]. They imitate evolution in time lapse. Each generation is formed by a number of individuals encoded in the form of binary character strings. These character strings are processed with the operators of evolution—selection, crossover and mutation to generate new binary character strings, which are selected according to their quality relative to the target function. For details about the theory of genetic algorithms and their application to bioprocesses see elsewhere [10–12].

Applied to the experiments discussed, the different nutrients and the temperature are the variables which are going to be varied. Within the variable space, a randomly generated ‘population’ of different media/temperature constellations (‘individuals’) forms the first generation. The results of the experiments in the first generation are evaluated according to the target function, which in this case is the 2-PE concentration in the organic phase. The best performing individual is taken over unchanged into the next generation, the others are allowed to mate and mutate and thus form new individuals for the next generation. This process is repeated until a suitable result is achieved or the result no longer significantly improves.

3. Experimental

3.1. Microorganism and chemicals

K. marxianus CBS 600 was obtained from the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands).

Methanol was HPLC gradient grade purchased from Roth (Karlsruhe, Germany). All other chemicals were purchased from Fluka (Buchs, Switzerland) in analytical grade save oleyl alcohol, which was technical grade.

3.2. Cultivation

Mineral, sugar and buffer solutions were heat sterilized for 20 min at 121 °C. The vitamin solutions were sterilized by filtration through 0.2 μm cellulose nitrate membrane filters (Whatman, Maidstone, England).

L-Phenylalanine was added in solid form without prior sterilization.

The original medium, which was used for the preparation of the freeze cultures and the inoculum, consisted of g/l: sucrose from molasses 30, L-phenylalanine 7, $MgSO_4$ 0.5, Na_2HPO_4 22.8, citric acid 10.3, YNB 0.17.

Erlenmeyer flasks with a total volume of 300 ml and a working volume of 100 ml (50 ml medium and 50 ml oleyl alcohol) were shaken on orbital shakers covered with heated hoods (B. Braun, Melsungen, Germany) at 180 rpm with an amplitude of 25 mm.

Inoculum was prepared by growing cells from a freeze culture (25%, w/w glycerol) for 8 h at 35 °C as described. After phase segregation in a separating funnel, the aqueous phase was transferred to a beaker where it was kept slightly in motion by a magnetic stir bar to prevent the cells from sedimenting.

Each medium was prepared in a measuring cylinder and distributed evenly into two shaking flasks to minimize standard deviation between parallel experiments. A volume of 49 ml medium was inoculated with 1 ml preculture, supplied with 50 ml oleyl alcohol and incubated for 24 h at the indicated temperature.

3.3. Sample preparation

Samples were taken after 24 h from the organic phase and centrifuged for 10 min. The supernatant was diluted 1:5 with methanol prior to HPLC analysis.

Table 2
Process variables used by the genetic algorithm

Variable	Initial value (non-optimized medium) (g/l)	Levels	Bit-string length	Lower limit[a] (g/l)	Upper limit[a] (g/l)
Molasses sucrose	30.0	31	5	0.0	30.0
Glucose	0.0			0.0	30.0
L-Phenylalanine	7.0	31	5	5.0	20.0
$MgSO_4$	0.5	11	4	0.0	1.0
KJ	0.00001	11	4	0.00001	0.001
Na_2MoO_4	0.00002			0.00002	0.002
Ca pantothenate	0.00004	11	4	0.00004	0.004
Niacin	0.00004	11	4	0.00004	0.004
Thiamine HCl	0.00004	11	4	0.00004	0.004
p-Aminobenzoic acid	0.00002			0.00002	0.002
Riboflavin	0.00002			0.00002	0.002
Biotin	0.0000002	11	4	0.0000002	0.00002
Myo-inositol	0.0002	11	4	0.0002	0.02
Pyrodoxin HCl	0.00004			0.00004	0.004
Temperature	35 °C	5	3	25 °C	45 °C

Values of the non-optimized medium before optimization and variable ranges design for the genetic algorithm. Grouped variables are indicated by grey background.
[a]To determine the specific lower and upper variable limits see detailed explanation in the text.

3.4. Genetic algorithm

The maximum number of individuals that could be handled in one generation was 40 shake flasks, corresponding to 20 parallel individuals. The number of variables should be equal to or less than half the number of individuals, thus some of the vitamins and minerals had to be merged into groups of two or three. For the groups see Table 2.

For each of the ten variables, the levels were set as shown in Table 2, resulting in a certain number of bits which add up to a bit string length of 41. The initial generation was generated at random. The probability of the occurrence of crossover was set at 95% and the mutation rate was set at 1%. Galop software (developed at the Institute of Biotechnology in Jülich, Germany, available from TeWiSo, Schwer-te, Germany) was used to execute the genetic algorithm calculations.

Table 2 shows the variable values of the non-optimized medium before and the variable ranges available during the optimization. For the vitamins and minerals the upper limit of the variable range was set two decimal powers above the values of the non-optimized medium as it was known that the concentrations chosen in former experiments were relatively low with 10% of the recommended YNB amount. Since L-phe is the precursor for aroma generation and it is known that the Ehrlich pathway is preferred at high L-phe concentrations, the lower limit was set at 5 g/l. The upper L-phe limit was confined by its maximum solubility of 26 g/l. The upper temperature limit was set at 45 °C as a thermotolerant yeast strain had been selected for the experiments. This was decided with regard to the technical scale process, where organophilic pervaporation, which is positively affected by an increase in temperature, is used as an in situ product removal technique.

For genetic algorithm experiments in shake flasks the initial sugar concentration has to be constant at a moderate level for all experiments to avoid oxygen limitation. Previous experiments had shown that 30 g/l was the optimal sugar concentration for *K. marxianus* CBS 600 [4]. The sugar can consist of 0–100% sucrose in the form of molasses with the complementary amount substituted by glucose.

4. Results and discussion

Fig. 2 shows the product concentrations of the experiments in generations 1–4 sorted by 2-PE concentration. The individuals of generation 4 are coloured differently to show that there was no significant increase from generation 3 to generation 4. Obviously the genetic algorithm came to a standstill at that point. With a maximum concentration of 4.0 g/l 2-PE in the organic phase the product concentration increased by 32% compared to the non-optimized medium.

4.1. Temperature effects

One thing however was noticeable in all four generations: the best results ocurred at 25 °C. To verify whether this was just a random effect due to the relatively small number of generations and low resolution of the parameter range for the temperature (five levels) or a physiologically caused move of the genetic algorithm towards the lower temperature limit, we tested the medium that had yielded the highest product concentration at temperatures between 22.5 and 42.5 °C in 2.5 °C increments. Furthermore the incubation time was extended to 39 h to ensure that even at low temperatures cultures would have reached the stationary phase, which might not be the case after 24 h.

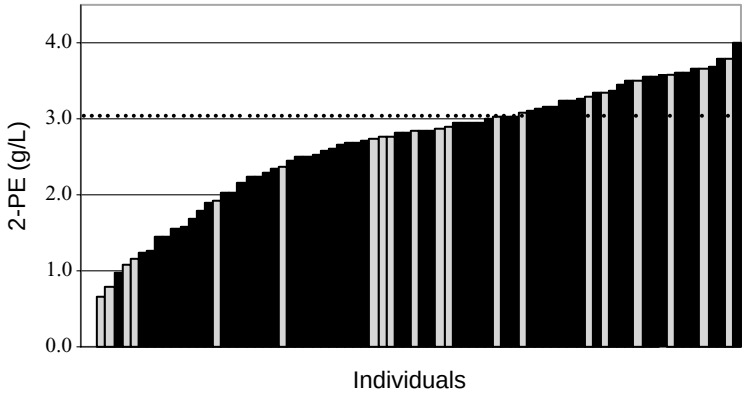

Fig. 2. 2-PE concentration of individuals in generations 1–4. Generation 4 in grey. The dotted line indicates the maximum concentration before optimization.

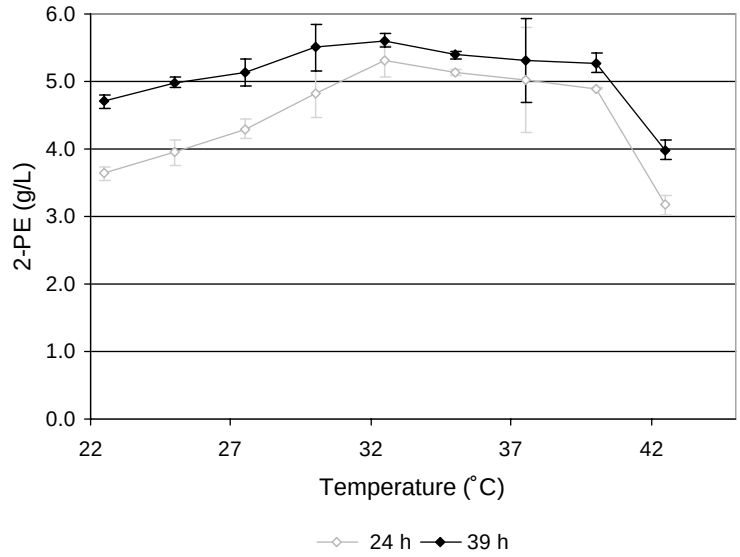

Fig. 3. Results of 'generation 5': temperature screening with *Kluyveromyces marxianus* CBS 600 in the best medium composition.

The results are shown in Figs. 3 and 4 as "generation 5" although strictly speaking they do not belong to the genetic algorithm.

Fig. 3 shows that the indications had been right. The temperature optimum for bioconversion with *K. marxianus* CBS 600 in the optimized medium is around 33 °C. At temperatures above 30 °C about 10% of the product is synthesized in the final 15 h, while below 30 °C this value is 20%.

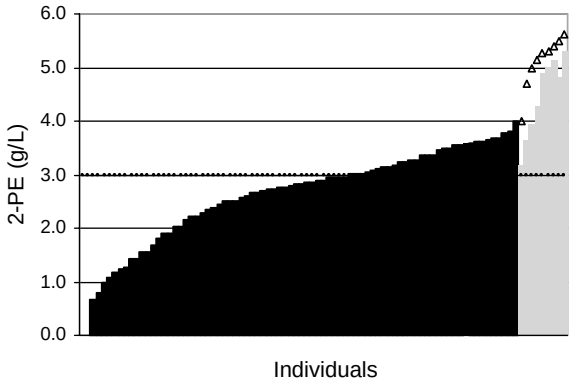

Fig. 4. 2-PE concentration of individuals in generations 1–5. 'Generation 5' in grey. The dotted line indicates the maximum concentration before optimization. Triangles indicate concentration of 'generation 5' individuals after 39 h.

Fig. 4 impressively demonstrates the positive effect of the additional temperature variation experiment on the final product concentration using the best medium identified by the genetic algorithm. The best individual yielded a 2-PE concentration of 5.3 g/l which rose to 5.6 g/l after 39 h. While the genetic algorithm already obtained a 32% product increase compared to the non-optimized medium, the temperature variation added another 45% or even 55% if the 39 h value is taken for the calculation. This adds up to a total improvement of product formation of 77 and 87%, respectively.

4.2. Vitamins and minerals

In Table 3 the composition of the non-optimized medium compared to the optimized medium is listed. The factor shows that while the concentrations of L-phenylalanine and

Table 3
Original and optimized culture conditions

Parameter	Non-optimized (g/l)	Optimized (g/l)	Factor
Molasses sucrose	30.0	8.0	0.3
Glucose	0.0	22.0	
L-Phenylalanine	7.0	9.0	1.3
$MgSO_4$	0.5	0.5	1
KJ	0.00001	0.0002	20
Na_2MoO_4	0.00002	0.0004	20
Ca pantothenate	0.00004	0.00296	74
Niacin	0.00004	0.0024	60
Thiamine HCl	0.00004	0.0032	80
p-Aminobenzoic acid	0.00002	0.0016	80
Riboflavin	0.00002	0.0016	80
Biotin	0.0000002	0.000016	80
Myo-inositol	0.0002	0.0160	80
Pyrodoxin HCl	0.00004	0.0032	80
Temperature	30 °C	32.5 °C	

$MgSO_4$ could be taken over nearly unchanged from the original medium, much higher doses of the minerals and especially the vitamins were needed.

4.3. Molasses

Fig. 5 shows the product concentrations obtained by all individuals in generations 1–5 versus their molasses sucrose sugar proportion.

In the optimal medium 27% of the total sugar concentration came from molasses. There was, however, a second local optimum towards 100% molasses sucrose derived sugar.

The best medium containing 30 g/l sugar solely supplied by molasses sucrose ('high molasses medium') yielded 3.28 g/l 2-PE and was identical in vitamin and mineral composition to the optimal, 27% molasses sucrose medium ('low molasses medium'). By undertaking the same temperature screening as with the low molasses medium it was possible to directly deduce effect of molasses on the product formation. The temperature optimum was found to be 32.5 °C with concentrations of 3.88 g/l 2-PE after 24 h and 4.40 g/l 2-PE after 39 h.

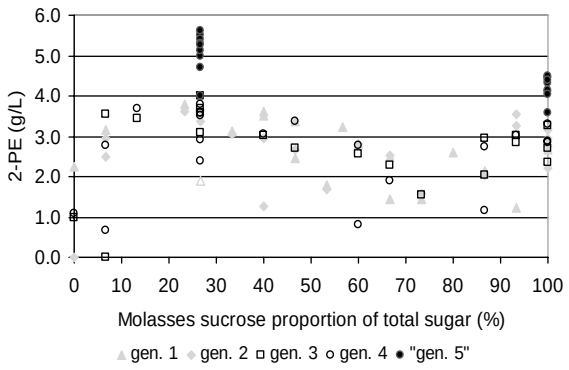

Fig. 5. Product concentrations of individuals in generations 1–5 related to the molasses sucrose proportion of total sugar (30 g/l).

In this case the genetic algorithm yielded a 9% product increase compared to the non-optimized medium. With the temperature variation the total improvement of product formation adds up to 29% (24 h) and 47% (39 h), respectively.

Obviously the high molasses content had a negative influence on product formation when compared with the low molasses medium, possibly the high amounts of potassium and iron were detrimental for the yeasts. On the other hand there was a significant improvement in product formation compared to the non-optimized medium, meaning that the raised vitamin/mineral content could compensate the negative molasses effect.

5. Conclusions

The number of theoretically possible medium compositions in this set of experiments is $>9 \times 10^{10}$.

With only 89 experiments an 87% increase in product concentration was achieved compared to the non-optimized medium. A concentration of 5.6 g/l 2-PE corresponds to 0.83 of the maximum theoretical yield when 9 g/l L-phenylalanine is present in the medium. Complete conversion is not to be expected under the suboptimal conditions that prevail in a shake flask experiment.

These facts considered lead to the conclusion that a genetic algorithm is an excellent means of optimizing of a fermentation medium. The fact that we deviated from the algorithm after generation 4 does not contradict this statement. Given a higher temperature resolution and thus more levels in the temperature range, the genetic algorithm would have come to the same result in the course of a few generations. This temperature aspect was so obvious that it was possible to shorten the medium development time by setting up the final generation externally, thereby belatedly correcting the experimental design of the genetic algorithm.

A characteristic drawback of genetic algorithms exploring large variable spaces is the question whether the local optimum found is also the global optimum under the given experimental conditions. Even with 0.83 of the maximum theoretical yield reached, possibly a medium composition exists that reaches an even higher product concentration. However, the efforts to find the global optimum would probably exceed the small increase that might be gained.

Further optimization might be achieved (with a corresponding higher experimental workload) by resolving the three pairs of variables and the triplet into separate variables and testing them together with a higher resolved temperature range around 33 °C.

Since we already started on a relatively high product concentration level with the non-optimized medium (about 3 g/l) the achievable percentage of improvement, which could be reached in this experimental setup, was limited.

The constant initial sugar concentration also impeded higher product concentrations in cases where the genetic algorithm had selected high precursor concentrations as the product formation is growth associated and therefore dependent on the availability of a C-source. Applying the genetic algorithm to a fed batch system in a bioreactor with the productivity as target function could circumvent this effect and might further improve the overall performance of this bioprocess.

The genetic algorithm revealed that the yeast strain needed up to an 80-fold higher dose of vitamins than initially supplied, indicating a significantly higher metabolic stress under bioconversion conditions. From an economic point of view it is highly interesting, that the genetic algorithm also indicated a second-best medium composition based solely on molasses derived sugar although the costs of the medium were not part of our target function, which was only defined as 'maximum final product concentration'. Nevertheless, such a high molasses medium is desirable because of its lower price. Whether the product loss compared to the low molasses medium can be made up for by a cheaper medium will be worked out in an economic analysis of the process if the results can be successfully scaled up from shaking flask to bioreactor.

This work exemplifies the fact that product yields in enzyme and whole-cell biocatalysis can be significantly raised not only by enzyme but also by medium engineering using a genetic algorithm even in the presence of complex medium components and starting from a pre-optimized system. Furthermore, unpredictable phenomena may be revealed by implementing medium engineering at an early research stage, as in this work the high impact of vitamins on the biocatalyst's performance under the process conditions needed.

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