A computational framework to identify metabolic engineering strategies for the co-production of metabolites

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ABSTRACT

Microbial production of chemicals is a more sustainable alternative to traditional chemical processes. However, the shift to bioprocess is usually accompanied by a drop in economic feasibility. Co-production of more than one chemical can improve the economy of bioprocesses, enhance carbon utilization and also ensure better exploitation of resources. While a number of tools exist for *in silico* metabolic engineering, there is a dearth of computational tools that can co-optimize the production of multiple metabolites. In this work, we propose an eXtended version of Flux Scanning based on Enforced Objective Flux (XFSEOF), identify intervention strategies to co-optimize for a set of metabolites. XFSEOF can be used to identify all pairs of products that can be co-optimized with ease, by a single intervention. Beyond this, it can also identify higher-order intervention strategies for a given set of metabolites. We have employed this tool on the genome-scale metabolic models of *Escherichia coli* and *Saccharomyces cerevisiae,* and identified intervention targets that can co-optimize the production of pairs of metabolites under both aerobic and anaerobic conditions. Anaerobic conditions were found to support the co-production of a higher number of metabolites when compared to aerobic conditions in both organisms. The proposed computational framework will enhance the ease of study of metabolite co-production and thereby aid the design of better bioprocesses.

Keywords: metabolic modelling, genome-scale models, constraint-based modelling, co-synthesis bioprocess, bioproduction, concomitant production

1 Introduction

Recent years have seen several advances in the usage of bioprocessing to produce a wide range of chemicals (1). Microorganisms can produce diverse and complex products from simple carbon sources. Nevertheless, there are many challenges in designing economically feasible bioprocesses. The advancements in synthetic biology have enabled the metabolic engineering of organisms to improve yield and productivity (2). Various computational strain design algorithms have been developed to identify the genetic manipulations required to over-produce a single product (3–5). Despite the increase in yield achieved through such rational strain design, the bioprocesses are unable to compete with the traditional chemical processes in many cases (6). This is due to two main reasons – (i) the cost of raw materials (ii) the maximum yield achievable for a given product in a given organism and environment is limited by the number of genetic manipulations that can be successfully implemented in a single strain (7). The cost of raw materials can be reduced by using agricultural waste as feedstock instead of a synthetic nutrient medium. The latter can be overcome by co-producing multiple products in the same bioprocess (8).

Co-production equips us to exploit the system in a better fashion and produce more valuable products from the same raw materials. A high-value, low-volume chemical can be co-produced with a low-value, high-volume product in order to increase the economic feasibility, as in the case of riboflavin and butanol, respectively (6). Co-production is also beneficial when we need to co-optimize a cocktail of metabolites rather than a single metabolite, as in the case of biofuels and fatty acids (9). It can also balance the carbon metabolism, as in the case of uridine and acetoin (10). High carbon inflow towards uridine causes excess production of acetate, which hampers the growth of the organism. Conversion of acetate to acetoin prevents over-acidification of the nutrient medium and thereby improves growth and uridine production. There are many studies that have successfully achieved co-production of a variety of products with/without genetic manipulation of the organisms. Polyhydroxyalkanoates are one of the common class of metabolites that are co-produced with other metabolites (11–13). Butanol and hydrogen have been co-produced in *Clostridium beijerinckii* (14), and ethanol and xylitol have been co-produced in *Candida tropicalis* (15,16). The carbon source, nutrient medium, pH etc., are optimized in such cases to improve the yield of metabolites. Metabolic engineering can further expand the number of products that are co-produced and also improve their yield significantly. Multiple metabolites like ethanol, isopropanol, butanol and 2,3 butanediol have been co-produced by optimizing the acetone-butanol-ethanol (ABE) fermentation pathway in *Clostridium acetobutylicum* (17). Nisin and 3-phenyllactic acid, two antimicrobial agents, were co-produced in *Lactococcus lactis* through genetic manipulation (18). Non-native metabolites can also be co-produced with other

metabolites, as in the case of butanol and riboflavin, by engineering the heterologous pathway in *C. acetobutylicum* (6).

Although many strain design algorithms have been successfully employed for metabolically engineering organisms to optimize a single product (19,20), few studies have applied it for co-production. The studies listed above only use existing literature and readily apparent deletion targets to achieve co-production. This limits the robustness of the bioprocesses that are designed. There is a lack of algorithms that can be easily applied to study co-production. In this study, we have extended the Flux Scanning based on Enforced Objective Flux (FSEOF) (21) algorithm to study co-production. Further, while deletion targets can be obtained for metabolites independently using existing algorithms like OptKnock (3), OptGene (4), there are very few algorithms that can identify amplification targets (22). In order to identify amplification targets in addition to knock-out targets for co-optimizing a set of metabolites, we propose a new methodology, eXtended (XFSEOF), adapting the FSEOF algorithm. XFSEOF has a simple computational framework that can be easily modified, and it also provides the entire set of potential intervention strategies in a single run while many algorithms are sequential, returning one intervention target per run. The utility of the potential intervention strategies obtained was further assessed using Flux Variability Analysis (FVA). We applied XFSEOF to evaluate all possible pairs of secretory metabolites in *Escherichia coli* and *Saccharomyces cerevisiae.* The different pairs of metabolites that can be co-produced through a single reaction deletion or amplification were obtained. This analysis helps us choose favorable pairs of metabolites for which higher-order intervention strategies can be obtained. We have demonstrated this by identifying the amplification targets, knock-out targets and mixed intervention strategies of size up to three to co-optimize the production of isobutanol and succinic acid in *S. cerevisiae.* Higher-order intervention strategies were able to achieve better yield with very little reduction in growth rate. Overall, our analyses provide an overall picture of the biosynthetic capabilities of an organism, particularly highlighting key interdependencies in metabolism.

2 Methods

2.1 Flux Balance Analysis (FBA)

FBA is a widely used steady-state constraint-based modelling approach to predict the metabolic capabilities of a variety of organisms (23–25). The metabolic network of an organism, which comprises all reactions known to occur in the organism, is represented as a stoichiometric matrix S, of size $m \times n$, where m is the number of metabolites and n is the number of reactions (Fig 1a). The entries in the jth column of S represent the stoichiometric coefficients of the metabolites that participate in the jth reaction. The minimum and maximum values of flux that any reaction can

assume are constrained by the lower and upper bounds, respectively. The flux through a reaction under a given set of conditions, at steady-state, is calculated by solving a linear programming (LP) problem. The LP problem is formulated as:

$$max_v \quad \boldsymbol{c}^T \boldsymbol{v}$$

$$\text{subject to} \quad \boldsymbol{S}.\boldsymbol{v} = \boldsymbol{0}$$

$$\text{while} \quad v_{l,i} \leq v_i \leq v_{u,i}\ ,\ \forall i \in [1, n]$$

where c is a vector of weights denoting the contribution of each of the n reactions to the objective function, $\boldsymbol{v} \epsilon R^n$ is the vector of metabolic fluxes, $\boldsymbol{v_l}$ and $\boldsymbol{v_u}$ are vectors representing the lower and upper bounds for the reaction fluxes, respectively (25).

2.2 Flux Variability Analysis (FVA)

FVA is used to identify the range of fluxes of each reaction that still satisfy the constraints, where two optimization problems are solved for each flux v_i of interest.

$$v_{j,max}\ /\ v_{j,min} = max_v\ /\ min_v \quad v_j$$

$$\text{s.t.} \quad \boldsymbol{S}.\boldsymbol{v} = \boldsymbol{0}$$

$$\text{while} \quad v_{l,i} \leq v_i \leq v_{u,i}\ , \forall i \in [1, n]$$

where $v \epsilon R^n$ is the vector of metabolic fluxes, $v_{j,max}$ and $v_{j,min}$ are the maximum and minimum values of fluxes, respectively for each reaction flux v_j (26).

2.3 Flux Scanning based on Enforced Objective Flux (FSEOF)

FSEOF (21) is a method used to identify potential reaction deletion and amplification targets in metabolic networks by observing the change in the reaction fluxes when the system moves from the wild-type flux of the target product to the theoretical maximum flux of the product (Fig 1b). The maximum biomass $v_{\max,bio}$ and maximum product $v_{\max,prdt}$ fluxes are obtained by performing FBA with the biomass reaction and the exchange reaction of the product as the objective, respectively. The flux of the target reaction, v_{prdt} is pinned to $x\%\ of\ v_{\max,prdt}$ ($x = 0 \rightarrow 100$). The change in the flux of a reaction, v_j, is studied as the product flux, v_{prdt} is increased, and it is classified as a potential deletion or amplification target based on the decrease or increase in its flux, respectively. The reactions that undergo no change or oscillations in the fluxes are discarded from the set of potential intervention strategies. The set of potential intervention strategies obtained are assessed by simulating each intervention and performing FVA on the mutant.

129 **2.4 XFSEOF: Extending FSEOF for co-optimization**

130 The Genome-Scale Metabolic Models (GSMMs) of *E. coli* iML1515 and *S. cerevisiae* iMM904 were
131 obtained from the BiGG models database (http://bigg.ucsd.edu/) (15). The simulations were
132 done with the following constraints on uptakes: -10 mmol/gDW/h glucose and -2 mmol/gDW/h
133 oxygen for aerobic conditions and -10 mmol/gDW/h glucose and zero oxygen uptake for
134 anaerobic conditions. The potential intervention strategies for all secretory metabolites
135 (metabolites that can be secreted into the medium) in the organism were obtained as described
136 in Section 2.3. Exchange and transport reactions were removed from the list of potential
137 intervention strategies to increase the relevance of the results and reduce the computational
138 time. The standard intervention strategies were obtained for all possible pairs of secretory
139 metabolites.

140 The reliability of the potential deletion targets was studied by deleting the reaction and
141 performing FVA for the product reaction. The reliability of the potential amplification targets was
142 studied by fixing the flux bound of the amplification target to its theoretical maximum and
143 performing FVA for the product reaction. Any reaction with more than a 5% increase in the
144 maximum product flux and less than 75% decrease in biomass flux is considered a promising
145 intervention strategy. FVA was performed using fastFVA to reduce the computational time (26).
146 To obtain higher-order intervention strategies, the potential targets obtained earlier for a given
147 set of metabolites were combined, and all possible combinations of intervention strategies of a
148 certain size (up to three) were evaluated using FVA. The score for each product i (total number
149 of products, n) and the overall score are calculated as

150 $$Score_i = \frac{(v_{prdt_i,mut} - v_{prdt_i,wt})}{(v_{bio,wt} - v_{bio,mut})}$$

151 $$Overall\ Score = \sum_{i=1}^{n} (Score_i)$$

152 where $v_{prdt_i,mut}$ and $v_{prdt_i,wt}$ are the mutant and wild-type maximum fluxes of the exchange
153 reaction of the product i, and $v_{bio,mut}$ and $v_{bio,wt}$ are the mutant and wild-type fluxes of the
154 biomass reaction. $Score_i$ denotes the score for the individual product while $overall\ score$
155 denotes the cumulative score for the set of metabolites. All simulations were performed in
156 MATLAB R2018a (MathWorks Inc., USA) using the COBRA Toolbox v3.0 (27) and IBM ILOG CPLEX
157 12.8 as the linear programming solver.

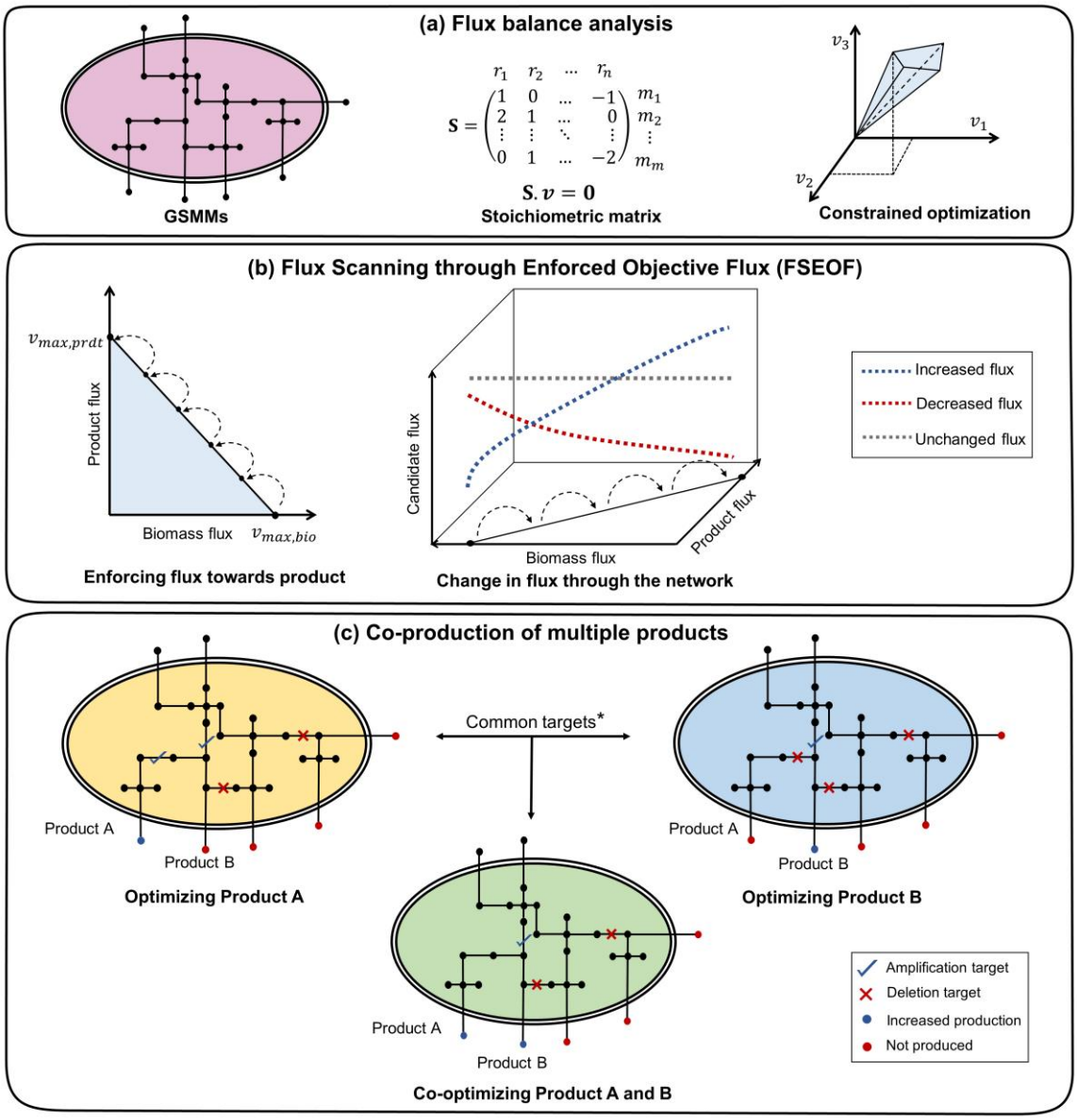

Figure 1. Workflow for XFSEOF. (a) The GSMM is represented as a stoichiometric matrix, which is used for FBA. (b) The flux through the product is increased in steps, and flux changes through all other reactions are studied. The reactions that have increased flux with an increase in product flux are potential amplification targets. The reactions that have decreased flux are potential deletion targets, while those with unchanged or oscillatory fluxes are excluded. (c) The targets common to products A and B are the potential targets for co-optimization. *The union of all potential targets for products A and B is used for higher-order intervention strategies

3 Results

Metabolic engineering strategies for the co-production of all pairs of secreted metabolites in *E. coli* and *S. cerevisiae* were obtained using XFSEOF as described in Section 2.4. We identified the intervention strategies required to optimize the co-production of metabolites in both aerobic and

anaerobic conditions. Anaerobic conditions favor the co-production of more pairs of metabolites when compared to aerobic conditions. The intervention strategy for each pair of metabolites is scored as in Section 2.4. The best intervention strategy can be chosen using the $overall\ score$. In cases where one metabolite might be favored over the others, the individual scores, $Score_i$ can be used to choose the best intervention strategies. Some of the intervention strategies obtained have been successfully verified through experimental studies in literature. This shows the credibility of the intervention strategies obtained. We discuss a few of the industrially significant metabolites and their intervention strategies, along with supporting literature. We also propose many other intervention strategies, which form a ready short-list for experimental validation. We were able to identify other hitherto unexplored intervention strategies, which may be better alternatives to those in existing literature, further demonstrating the utility of the algorithm.

3.1 Co-production in *Escherichia coli*

E. coli is one of the well-studied model organisms and has high-quality GSMMs available. The latest GSMM, *i*ML1515 (28), was used in this study, and the co-production of 337 secretory metabolites was studied in both aerobic and anaerobic conditions.

3.1.1 Aerobic fermentation

Co-production of all pairs of metabolites was studied in *E. coli* using XFSEOF and FVA as described in Sections 2.3 and 2.4. Out of ${}^{337}C_2$ pairs of secretory metabolites, only 237 could be successfully overproduced through deletion or amplification of a single reaction. The intervention strategies for a few industrially significant pairs of metabolites are listed in Table 1. One of the important pairs of metabolites that can be easily co-produced is L-lysine, a food additive and drug additive and cadaverine, which is essential for polyamide production. XFSEOF was able to identify several reactions from the diaminopimelate pathway (DAP), which can be over-expressed to co-produce L-lysine and cadaverine. An experimental study by Xu *et al*. (29) demonstrates the effect of engineering the DAP pathway in *E. coli* for the production of L-lysine. This indicates the reliability of the results obtained through computational methods. Another significant result is the co-production of succinate and ethanol through the amplification of glyceraldehyde-3-phosphate dehydrogenase. Other studies have successfully co-produced ethanol and succinate by other genetic manipulations (30).

3.1.2 Anaerobic fermentation

Anaerobic conditions support the co-production of more metabolites when compared to aerobic conditions. More than 1000 pairs of metabolites can be co-produced, out of which few are listed in Table 2. L-lysine and cadaverine can be co-produced under anaerobic conditions too. But the maximum flux achievable is lower when compared to aerobic conditions. The yield of metabolites

like acetate, formate and hexanoate can be co-optimized by deleting acetaldehyde dehydrogenase or alcohol dehydrogenase. We also found that succinate and lactate can be co-produced by the knock-out of pyruvate formate lyase. The effect of deletion of *pflB* gene encoding pyruvate formate lyase has been experimentally verified in *E. coli* for succinate production (31) and lactate production (32) through separate studies. This shows that there are multiple co-production strategies available in the existing literature that can be easily utilized to design an efficient process.

3.2 Co-production in *Saccharomyces cerevisiae*

Another industrially relevant and well-studied model organism is *S. cerevisiae.* Though heterologous pathways have not been analyzed in this study, one can easily modify the GSMM and apply XFSEOF to identify co-production strategies for heterologous metabolites. Since *S. cerevisiae* is a better candidate for recombinant protein production, it is essential to study co-production in yeast (33). It can also produce more complex metabolites when compared to *E. coli* and is, therefore, a favorable candidate for bio-production. The latest GSMM iMM904 (34) was used, and the ability to optimize the co-production of 164 secretory metabolites was studied in both aerobic and anaerobic conditions.

3.2.1 Aerobic fermentation

We found that many industrially important metabolites like ethanol and L-alanine, and 4-aminobutanoate and L-serine can be co-produced in *S. cerevisiae* under aerobic conditions. We were also able to co-optimize isobutyl alcohol and 2-methyl propanal, which are long-chain alcohols that are used as biofuels. The deletion of pyruvate dehydrogenase increases the production of pyruvate and acetate, as shown in Table 3. Although the deletion of pyruvate dehydrogenase has not been experimentally verified as yet, a similar study has been carried out in *E. coli* (35). In this study, it has been shown that the deletion of the genes encoding pyruvate dehydrogenase improves pyruvate production (35). In addition to pyruvate dehydrogenase, XFSEOF was able to identify several other amplification targets, which can also improve the production of pyruvate and acetate.

3.2.2 Anaerobic fermentation

As in the case of *E. coli,* anaerobic fermentation enables the co-production of more pairs of metabolites in *S. cerevisiae* when compared to aerobic fermentation. 2-methyl-1-butanol, which is an important solvent used in the manufacture of pesticides and paints and isobutyl alcohol, which is a biofuel, can be co-produced by the amplification of malic enzyme, as shown in Table 4. Formate, which is used in dyeing and printing, can be co-produced with spermidine, a metabolite increasingly studied for its anti-ageing properties (36), through the amplification of a number of

238 reactions. These strategies not only include readily apparent reactions that are involved in
239 spermidine synthesis like spermidine synthase and adenosylmethionine decarboxylase but also
240 provide some non-intuitive strategies like the amplification of aspartate transaminase or 2-keto-
241 4-methylthiobutyrate transaminase. We also found that the deletion of pyruvate decarboxylase
242 improves the production of succinate, isobutyl alcohol and pyruvate. The effect of deletion of
243 pyruvate decarboxylase has been studied in *S. cerevisiae,* and the improvement in the production
244 of pyruvate (37) and succinate (38) has been verified experimentally in separate studies in
245 literature.

246 **3.2.3 Higher-order intervention strategies – co-production of isobutanol and succinate**

247 Higher-order intervention strategies can increase the maximum yield achievable for any product
248 with a little difference in growth rate when compared to single interventions. But they are more
249 cumbersome to identify, as the problem becomes time-consuming and computationally
250 expensive. Instead of identifying higher-order targets for all metabolites in an organism, we have
251 used the previous analysis to explore the metabolic capabilities of the organism and chose one
252 set of metabolites to demonstrate the power of higher-order intervention strategies.

253 Isobutanol is a long-chain alcohol that is an attractive biofuel (39). Succinic acid is an important
254 metabolite essential for the production of various other products like biodegradable polymers,
255 fatty acids, butyrolactone and tetrahydrofuran (40). The co-production of isobutanol and
256 succinate has been proposed as a sustainable and economical process by Xu *et al.*, (41). They have
257 discussed the development of various strains for the production of isobutanol and succinate
258 separately. They emphasize how the co-production of isobutanol and succinate is not only of
259 economic significance, but the high amount of carbon dioxide released from long-chain alcohol
260 fermentation can be used for succinate production, and is hence also of ecological importance.
261 But the article does not discuss any strategy to co-optimize the production of isobutanol and
262 succinate.

263 Here we identified the higher-order intervention strategies (size up to three) for co-production
264 of isobutanol and succinate in *S. cerevisiae* in aerobic conditions. More than 3700 interventions
265 can improve the yield of both the metabolites when compared to the wild-type strain. Table 5
266 lists a few examples of each type of intervention strategy obtained, some of which are also found
267 in experimental studies in literature. The deletion of pyruvate decarboxylase has been shown to
268 improve the production of isobutanol by Kondo *et al.,* (42). Zahoor *et al.,* (38) have shown that
269 both pyruvate decarboxylase deletion and fumarase deletion can increase the production of
270 succinate. This shows the dependability of the results obtained using the algorithm.

271 **4 Discussion**

Chemical processes based on fossil fuels are cheaper when compared to bioprocesses, which leads to reluctance in the adoption of sustainable bioprocesses in industries. To improve the economic feasibility of a bioprocess, we can optimize the process variables and/or genetically engineer the microbes (43). Even then, in some cases, the bioprocess might be less lucrative when compared to their chemical counterparts (14). In such cases, we can co-produce multiple metabolites to improve the economic feasibility and efficiency of a bioprocess. Co-production also ensures better utilization of microbial capabilities, and better balance in the carbon metabolism. While there are multiple computational tools and algorithms to identify intervention strategies for a single product, there is a lack of readily appliable algorithms for co-production. As a result, almost no co-production study in existing literature was found to use computational algorithms to aid rational strain design. All of the studies rely on previous findings or readily apparent strategies to achieve co-production. This limits the intervention strategies designed.

In this study, we present XFSEOF, by adapting the effective FSEOF algorithm to study the co-optimization of a set of metabolites. FSEOF is a well-established constraint-based modelling algorithm, which has been used to reliably predict metabolic engineering strategies for a variety of systems (21,44–46). Extending FSEOF, we examined the co-production of multiple pairs of metabolites, and both deletion and amplification targets were obtained in *E. coli* and *S. cerevisiae* under both aerobic and anaerobic conditions. Anaerobic fermentation enabled the co-production of a higher number of metabolites when compared to aerobic fermentation in both organisms. This can be due to the incomplete respiration in the absence of oxygen that leads to the formation of multiple by-products. Also, *S. cerevisiae* produces more industrially significant metabolites when compared to *E. coli.* Some of these proposed intervention strategies have been verified experimentally by other studies in literature, as mentioned in Section 3. This shows the efficacy of the algorithm in furnishing reliable targets. XFSEOF also provides both intuitive and non-intuitive intervention strategies (as discussed in Section 3.2.2) for a set of metabolites, which exhibits the functionality of the algorithm.

The co-optimization analysis for all possible pairs of metabolites in the network is intended to be exploratory in order to give a larger picture of the metabolic capabilities of the organism. This analysis showed that around 200 pairs could be co-optimized in *E. coli* in aerobic conditions, and around 1000 pairs of metabolites could be co-optimized in all the other cases, respectively. Once a few sets of desirable metabolites are chosen from the previous analysis, XFSEOF can be extended to obtain higher-order intervention strategies, as shown in Section 3.2.3. Exploring the higher-order strategies can expand the efficiency of the intervention targets obtained. Since the evaluation of higher-order intervention strategies is laborious and computationally expensive, we have limited the size to a maximum of three manipulations at a time. It not only enhances

yield, but also provides alternate routes to achieve a similar yield. The advantageous strategies can be chosen based on the ease of manipulation in an experimental setup in such cases.

The evaluation of the results is carried out using FVA, which ensures the robustness of the targets obtained. While FBA provides one optimal solution from the solution space, FVA gives the entire range of values the flux can take up. This is a significant difference that sets XFSEOF apart from other existing algorithms like OptKnock. Also, the algorithm validates and returns all the intervention strategies in a single run, contrary to the existing algorithms, most of which are sequential and require a separate run for each strategy obtained. The set of intervention strategies validated through FVA can be short-listed for experimental verification using the scores. The $overall\ score$ can be used to compare the effectiveness of different intervention strategies. If one product is more favored than the others economically or otherwise, we can use the individual scores $Score_i$ to choose the appropriate strategy for the process that is formulated. The products can be chosen based on their economic value, or ease of co-production. One drawback of co-production is the cost associated with downstream processing. But this can be overcome by choosing easily separable products or choosing metabolites such that one is accumulated in the cell and one is secreted out, as in the case of polyhydroxy butyrate and succinate, respectively (47). XFSEOF not only identifies intervention strategies for co-production of a given set of metabolites, but allows us to explore the different combinations of products that can be co-produced in an easy and efficient manner.

5 Conclusion

Co-production can open new avenues for the sustainable production of chemicals. Designing bioprocesses for co-production using laboratory experiments alone is cumbersome and can result in sub-optimal strategies. XFSEOF empowers us to explore and exploit microbial systems in a better fashion. It can be used to computationally study and optimize co-production by identifying intervention strategies for multiple metabolites and thereby improve the efficiency of bioprocesses. It should be noted that the co-optimization analysis was limited to pairs of metabolites to reduce the computational time. But XFSEOF can be easily extended to co-optimize a more extensive set of metabolites. To conclude, this study can be used to identify various genetic manipulations that can co-optimize a set of products, which might be challenging to achieve through pure experimentation. It provides a novel and critical approach to study co-production computationally. We hope this study will aid the design and development of more sustainable bioprocesses.

Data availability

All models used in this work and the codes used for our analysis are available at https://github.com/RamanLab/XFSEOF

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474

475 **Tables**

476 **Table 1. Intervention strategies for co-production of pairs of metabolites in *E. coli* under aerobic conditions**

#	Product A	WT flux A	Product B	WT flux B	Intervention	Mutant product flux A	Mutant product flux B	Score A	Score B	Score A+B	Mutant biomass flux	KO/ Amp
1	L-lysine	*	1,5-Diamino pentane	*	Diaminopimelate decarboxylase	5.81	5.81	8.84	8.84	17.68	0.22	Amp
					Diaminopimelate epimerase	5.81	5.81	8.84	8.84	17.68	0.22	Amp
					Dihydrodipicolinate reductase	5.81	5.81	8.84	8.84	17.68	0.22	Amp
					Dihydrodipicolinate synthase	5.81	5.81	8.84	8.84	17.68	0.22	Amp
					Succinyl-diaminopimelate desuccinylase	5.81	5.81	8.84	8.84	17.68	0.22	Amp
					Tetrahydrodipicolinate succinylase	5.81	5.81	8.84	8.84	17.68	0.22	Amp
2	Succinate	*	Ethanol	*	Glyceraldehyde-3-phosphate dehydrogenase	12.86	15.84	19.56	24.10	43.67	0.22	Amp
3	Spermidine	*	5-Methylthio-D-ribose	*	Adenosylmethionine decarboxylase	2.01	2.01	3.06	3.06	6.12	0.22	Amp
					Methylthioadenosine nucleosidase	2.01	2.01	3.06	3.06	6.12	0.22	Amp
					Spermidine synthase	2.01	2.01	3.06	3.06	6.12	0.22	Amp
4	Xanthine	*	D-lactate	*	Glyceraldehyde-3-phosphate dehydrogenase	8.90	15.84	13.55	24.10	37.65	0.22	Amp
5	Glycine	*	L-aspargine	*	Glyceraldehyde-3-phosphate dehydrogenase	20.91	11.82	31.80	17.99	49.79	0.22	Amp
6	Fe-enterobactin	*	Enterobactin	*	2,3-dihydro- 2,3-dihydroxybenzoate dehydrogenase	1.29	1.29	1.97	1.97	3.93	0.22	Amp
					Isochorismatase	1.29	1.29	1.97	1.97	3.93	0.22	Amp
7	Pyruvate	*	L-aspargine	*	Glyceraldehyde-3-phosphate dehydrogenase	18.01	11.82	27.39	17.99	45.38	0.22	Amp

WT – Wild Type; * - Less than 10^{-5} mmol/gDW/h

477

478 **Table 2. Intervention strategies for co-production of pairs of metabolites in *E. coli* under anaerobic conditions**

#	Product A	WT flux A	Product B	WT flux B	Intervention	Mutant product flux A	Mutant product flux B	Mutant biomass flux	Score A	Score B	Score A+B	KO/ Amp
1	Acetate	8.83	Formate	18.22	Acetaldehyde dehydrogenase	18.18	36.79	0.12	237.95	472.31	710.26	KO
					Alcohol dehydrogenase	18.18	36.79	0.12	237.95	472.31	710.26	KO
2	Succinate	0.05	D-lactate	3.76E -04	Glyceraldehyde-3-phosphate dehydrogenase	14.98	19.41	0.04	126.78	164.92	291.71	Amp
					Acetaldehyde dehydrogenase	9.07	18.12	0.12	229.50	461.04	690.55	KO
					Alcohol dehydrogenase	9.07	18.12	0.12	229.50	461.04	690.55	KO
					Pyruvate formate lyase	0.49	17.76	0.12	10.57	426.65	437.22	KO
3	L-alanine	*	Xanthine	*	Glyceraldehyde-3-phosphate dehydrogenase	13.56	0.97	0.04	115.18	8.23	123.41	Amp
4	Spermidine	*	5-Methylthio-D-ribose	*	Adenosylmethionine decarboxylase	0.64	0.64	0.04	5.46	5.46	10.93	Amp
					Methylthioadenosine nucleosidase	0.64	0.64	0.04	5.46	5.46	10.93	Amp
					Spermidine synthase	0.64	0.64	0.04	5.46	5.46	10.93	Amp
5	L-aspartate	*	L-glutamate	*	Glyceraldehyde-3-phosphate dehydrogenase	6.03	4.52	0.04	51.19	38.39	89.59	
6	L-lysine	*	1,5-Diamino pentane	*	Diaminopimelate decarboxylase	2.72	2.72	0.04	23.04	23.04	46.07	Amp
					Diaminopimelate epimerase	2.72	2.72	0.04	23.04	23.04	46.07	Amp
					Dihydrodipicolinate synthase	2.72	2.72	0.04	23.04	23.04	46.07	Amp
					Succinyl-diaminopimelate desuccinylase	2.72	2.72	0.04	23.04	23.04	46.07	Amp
					Tetrahydrodipicolinate succinylase	2.72	2.72	0.04	23.04	23.04	46.07	Amp
7	Acetate	8.83	Hexanoate	*	Acetaldehyde dehydrogenase	18.18	4.53	0.12	237.95	115.26	353.21	KO
					Alcohol dehydrogenase	18.18	4.53	0.12	237.95	115.26	353.21	KO

WT – Wild Type; * - Less than 10^{-5} mmol/gDW/h

479

480 **Table 3. Intervention strategies for co-production of pairs of metabolites in *S. cerevisiae* under aerobic conditions**

#	Product A	WT flux A	Product B	WT flux B	Intervention	Mutant product flux A	Mutant product flux B	Mutant biomass flux	Score A	Score B	Score A+B	KO/ Amp
1	Ethanol	15.81	L-alanine	1.69E -04	Sedoheptulose 1,7-bisphosphate D-glyceraldehyde-3-phosphate-lyase	18.45	0.15	0.07	12.22	0.71	12.93	Amp
					Phosphofructokinase (s7p)	18.45	0.15	0.07	12.22	0.71	12.93	Amp
2	Acetate	3.55E -03	2,3 butanediol	3.2E-04	Pyruvate dehydrogenase	1.48	0.25	0.28	230.94	38.48	269.42	KO
					Enolase	10.71	10.27	0.07	49.61	47.61	97.22	Amp
					Fructose-bisphosphate aldolase	10.70	10.27	0.07	49.62	47.64	97.26	Amp
					Glyceraldehyde-3-phosphate dehydrogenase	10.71	10.27	0.07	49.61	47.61	97.22	Amp
					Triose-phosphate isomerase	10.70	10.27	0.07	49.62	47.64	97.26	Amp
3	Isobutyl alcohol	*	Succinate	*	Pyruvate decarboxylase	7.63	5.45	0.19	79.29	56.66	135.95	KO
					Enolase	9.56	12.97	0.07	44.30	60.10	104.40	Amp
					Glyceraldehyde-3-phosphate dehydrogenase	9.56	12.97	0.07	44.30	60.13	104.43	Amp
					Triose-phosphate isomerase	9.56	12.97	0.07	44.33	60.17	104.50	Amp
4	2-methylpropanal	*	Isobutyl alcohol	*	3 Methyl 2 oxobutanoate decarboxylase	6.50	9.36	0.07	30.14	43.39	73.54	Amp
					Acetolactate synthase mitochondrial	6.50	9.36	0.07	30.14	43.40	73.54	Amp
					Dihydroxy acid dehydratase 2 3 dihydroxy 3 methylbutanoate mitochondrial	6.50	9.36	0.07	30.14	43.40	73.54	Amp
					Enolase	8.95	9.56	0.07	41.47	44.30	85.77	Amp
					Glyceraldehyde-3-phosphate dehydrogenase	8.95	9.56	0.07	41.47	44.30	85.77	Amp
					Acetohydroxy acid isomeroreductase mitochondrial	6.50	9.36	0.07	30.14	43.40	73.54	Amp
					Triose-phosphate isomerase	8.94	9.56	0.07	41.49	44.33	85.82	Amp
5	L-Glutamate	*	2-Oxoglutarate	*	Citrate synthase	2.93	1.95	0.07	13.58	9.05	22.63	Amp
					Enolase	4.50	4.04	0.07	20.85	18.73	39.58	Amp

					Fructose-bisphosphate aldolase	4.50	4.04	0.07	20.86	18.73	39.60	Amp
					Glyceraldehyde-3-phosphate dehydrogenase	4.50	4.04	0.07	20.85	18.73	39.58	Amp
					Isocitrate dehydrogenase	2.99	2.00	0.07	13.87	9.25	23.12	Amp
					Triose-phosphate isomerase	4.50	4.04	0.07	20.86	18.73	39.60	Amp
6	Acetate	3.55E-03	Pyruvate	3.24E-04	Pyruvate dehydrogenase	1.48	0.25	0.28	230.94	38.48	269.42	KO
					Aspartate-semialdehyde dehydrogenase	8.79	11.85	0.07	41.14	55.49	96.63	Amp
					Enolase	10.71	14.42	0.07	49.61	66.83	116.44	Amp
					Fructose-bisphosphate aldolase	10.70	14.41	0.07	49.62	66.85	116.47	Amp
					Glyceraldehyde-3-phosphate dehydrogenase	10.71	14.42	0.07	49.61	66.83	116.44	Amp
					Triose-phosphate isomerase	10.70	14.41	0.07	49.62	66.85	116.47	Amp
7	4-Aminobutanoate	*	L-serine	*	Glyceraldehyde-3-phosphate dehydrogenase	4.77	6.98	0.07	22.10	32.34	54.44	Amp
					Triose-phosphate isomerase	4.77	6.97	0.07	22.11	32.35	54.45	Amp
8	L-alanine	1.69E-04	L-cysteine	*	Glucose 6-phosphate dehydrogenase	10.60	1.20	0.07	49.11	5.56	54.67	Amp
					Phosphogluconate dehydrogenase	10.60	1.20	0.07	49.11	5.56	54.67	Amp
					6-phosphogluconolactonase	10.60	1.20	0.07	49.11	5.56	54.67	Amp
					Ribulose 5-phosphate 3-epimerase	10.60	1.20	0.07	49.11	5.56	54.67	Amp
					Transketolase	10.60	1.20	0.07	49.12	5.56	54.68	Amp
					Ribose-5-phosphate isomerase	18.16	2.21	0.08	85.44	10.40	95.84	Amp
9	Sn-Glycero-3-phosphocholine	*	L-methionine	*	Methionine synthase	0.02	2.19	0.07	0.11	10.17	10.27	Amp
					5 10 methylene-tetrahydrofolatereductase NADPH	0.02	2.19	0.07	0.11	10.17	10.27	Amp
					Ribose-5-phosphate isomerase	0.66	1.71	0.08	3.11	8.03	11.14	Amp
10	2 methylbutyl acetate	*	2-Methyl-1-butanol	*	Ribose-5-phosphate isomerase	3.00	4.42	0.08	14.13	20.78	34.92	Amp
WT – Wild Type; * - Less than 10^{-5} mmol/gDW/h												

481

482 **Table 4. Intervention strategies for co-production of pairs of metabolites in *S. cerevisiae* under anaerobic conditions**

#	Product A	WT flux A	Product B	WT flux B	Intervention	Mutant product flux A	Mutant product flux B	Mutant biomass flux	Score A	Score B	Score A+B	KO/ Amp
1	2-Methyl-1-butanol	*	Isobutyl alcohol	*	Malic enzyme NADP mitochondrial	0.03	9.64	0.05	0.20	61.30	61.51	Amp
2	Isobutyl alcohol	*	Pyruvate	*	Pyruvate decarboxylase	8.73	5.66	0.11	88.74	57.57	146.31	KO
					Enolase	9.68	9.97	0.05	61.59	63.45	125.03	Amp
					Fructose-bisphosphate aldolase	9.68	9.96	0.05	61.67	63.47	125.14	Amp
					Glyceraldehyde-3-phosphate dehydrogenase	9.68	9.97	0.05	61.60	63.45	125.05	Amp
					Triose-phosphate isomerase	9.68	9.96	0.05	61.66	63.47	125.13	Amp
3	Formate	6.26E-04	Spermidine	*	Adenosylmethionine decarboxylase	1.22	1.22	0.05	7.76	7.76	15.52	Amp
					Aspartate transaminase	1.21	1.21	0.05	7.75	7.76	15.51	Amp
					2 3 diketo 5 methylthio 1 phosphopentane degradation	1.22	1.22	0.05	7.76	7.76	15.52	Amp
					5-Methylthio-5-deoxy-D-ribulose 1-phosphate dehydratase	1.22	1.22	0.05	7.76	7.76	15.52	Amp
					5 methylthioadenosine phosphorylase	1.22	1.22	0.05	7.76	7.76	15.52	Amp
					5-methylthioribose-1-phosphate isomerase	1.22	1.22	0.05	7.76	7.76	15.52	Amp
					Spermidine synthase	1.22	1.22	0.05	7.76	7.76	15.52	Amp
					2-keto-4-methylthiobutyrate transamination	1.22	1.22	0.05	7.76	7.76	15.52	Amp
4	4-Amino butanoate	*	Isobutyl acetate	*	Enolase	2.84	2.91	0.05	18.04	18.50	36.53	Amp
					Fructose-bisphosphate aldolase	2.84	2.90	0.05	18.11	18.50	36.61	Amp
					Glyceraldehyde-3-phosphate dehydrogenase	2.85	2.91	0.05	18.11	18.50	36.61	Amp
					Triose-phosphate isomerase	2.84	2.90	0.05	18.11	18.50	36.61	Amp

5	2-Methyl-1-butanol	*	Glycine	*	Aspartate kinase	0.05	4.55	0.05	0.32	28.95	29.26	Amp
					Glucose 6-phosphate dehydrogenase	0.08	4.15	0.05	0.52	26.39	26.91	Amp
					Phosphogluconate dehydrogenase	0.08	4.15	0.05	0.52	26.39	26.91	Amp
					Homoserine dehydrogenase NADH irreversible	0.05	4.55	0.05	0.32	28.95	29.26	Amp
					Homoserine kinase	0.05	4.55	0.05	0.32	28.91	29.22	Amp
					6-phosphogluconolactonase	0.08	4.15	0.05	0.52	26.39	26.91	Amp
					Ribulose 5-phosphate 3-epimerase	0.08	4.15	0.05	0.52	26.39	26.91	Amp
					Ribose-5-phosphate isomerase	3.31	4.01	0.06	21.52	26.04	47.56	Amp
					Threonine synthase	0.05	4.55	0.05	0.32	28.91	29.22	Amp
					Transketolase	0.08	4.15	0.05	0.52	26.39	26.91	Amp
					Transketolase	0.08	4.15	0.05	0.52	26.39	26.91	Amp
6	Isobutyl alcohol	*	Succinate	0.67	Pyruvate decarboxylase	8.73	6.70	0.11	88.74	61.27	150.02	KO
7	L-Glutamate	*	Xanthine	*	Fructose-bisphosphate aldolase	2.81	0.95	0.05	17.90	6.04	23.94	Amp
					Glyceraldehyde-3-phosphate dehydrogenase	2.81	0.95	0.05	17.90	6.04	23.93	Amp
					Triose-phosphate isomerase	2.81	0.95	0.05	17.90	6.04	23.94	Amp
8	Sorbitol	*	L-methionine	*	Ribose-5-phosphate isomerase	5.61	1.28	0.06	36.45	8.29	44.74	Amp
9	2,3 butanediol	*	L-serine	*	Fructose-bisphosphate aldolase	9.30	4.74	0.05	59.21	30.19	89.40	Amp
					Glyceraldehyde-3-phosphate dehydrogenase	9.30	4.74	0.05	59.19	30.19	89.37	Amp
					Triose-phosphate isomerase	9.30	4.74	0.05	59.21	30.19	89.40	Amp
WT – Wild Type; * - Less than 10^{-5} mmol/gDW/h												

483

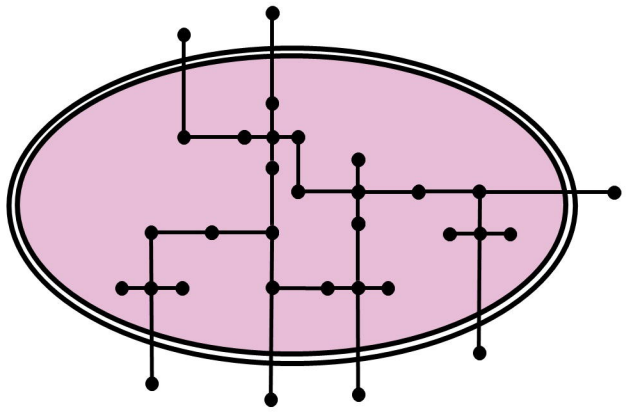
484 **Table 5. Higher-order intervention strategies for co-production of isobutanol and succinate in *S. cerevisiae* under aerobic conditions**

#	Intervention 1	Intervention 2	Intervention 3	A/K/AK/ AKK/AAK	Mutant flux 1	Mutant flux 2	Biomass flux	Score A	Score B	Score A+B
1	Glyceraldehyde-3-phosphate dehydrogenase	Pyruvate kinase	NA	A	7.19	8.60	0.20	81.73	97.87	179.59
2	Enolase	Pyruvate kinase	NA	A	7.13	8.60	0.20	81.08	97.87	178.95
3	Glutamate 5-kinase	Phosphoglycerate dehydrogenase	Pyruvate decarboxylase	K	7.96	5.80	0.19	79.81	58.12	137.93
4	Fumarase	Phosphoserine phosphatase	Pyruvate decarboxylase	K	7.95	5.78	0.19	79.78	58.05	137.84
5	Aldehyde dehydrogenase	Glycerol 3 phosphate dehydrogenase	NA	AK	2.99	2.52	0.22	43.76	36.77	80.54
6	Succinate CoA ligase ADP forming	Pyruvate decarboxylase	NA	AK	7.63	5.45	0.19	79.31	56.69	136.00
7	Acetolactate synthase	Oxoglutarate dehydrogenase lipoamide	Guanylate kinase	AAK	4.72	6.89	0.15	34.51	50.38	84.89
8	Citrate synthase	Dihydroxy-acid dehydratase	Prephenate dehydrogenase	AAK	4.73	6.98	0.14	32.31	47.71	80.02
9	Aldehyde dehydrogenase	Ribonucleoside-diphosphate reductase	Ribulose 5-phosphate 3-epimerase	AKK	2.99	2.48	0.22	43.84	36.32	80.16
10	Succinate CoA ligase ADP forming	Phosphoserine phosphatase	Pyruvate decarboxylase	AKK	7.95	5.78	0.19	79.78	58.05	137.84

A- Amplification of all reactions; K- Knock-out of all reactions; AK – Amplification+ Knock-out; AAK – Amplification + Amplification + Knock-out; AKK – Amplification + Knock-out+ Knock-out

485

(a) Flux balance analysis

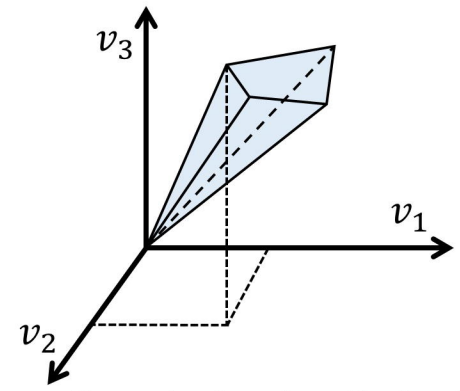


GSMMs

$$\begin{array}{cc} & \begin{array}{cccc} r_1 & r_2 & \dots & r_n \end{array} \\ \mathbf{S} = & \begin{pmatrix} 1 & 0 & \dots & -1 \\ 2 & 1 & \dots & 0 \\ \vdots & \vdots & \ddots & \vdots \\ 0 & 1 & \dots & -2 \end{pmatrix} \begin{array}{c} m_1 \\ m_2 \\ \vdots \\ m_m \end{array} \end{array}$$

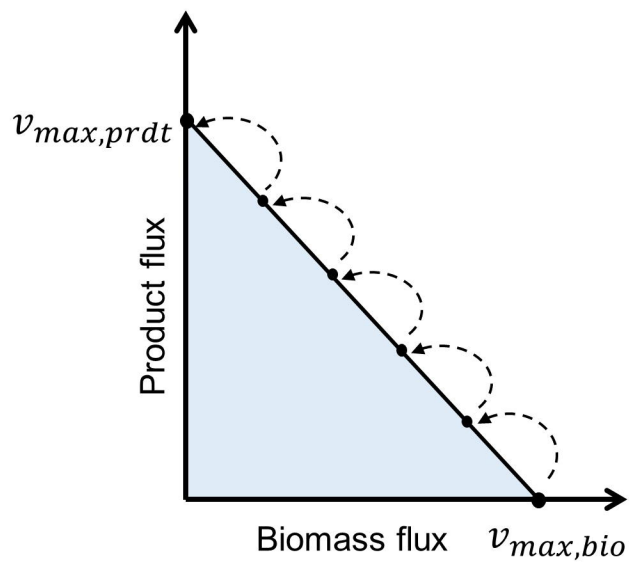
$$\mathbf{S}.\boldsymbol{v} = \mathbf{0}$$

Stoichiometric matrix

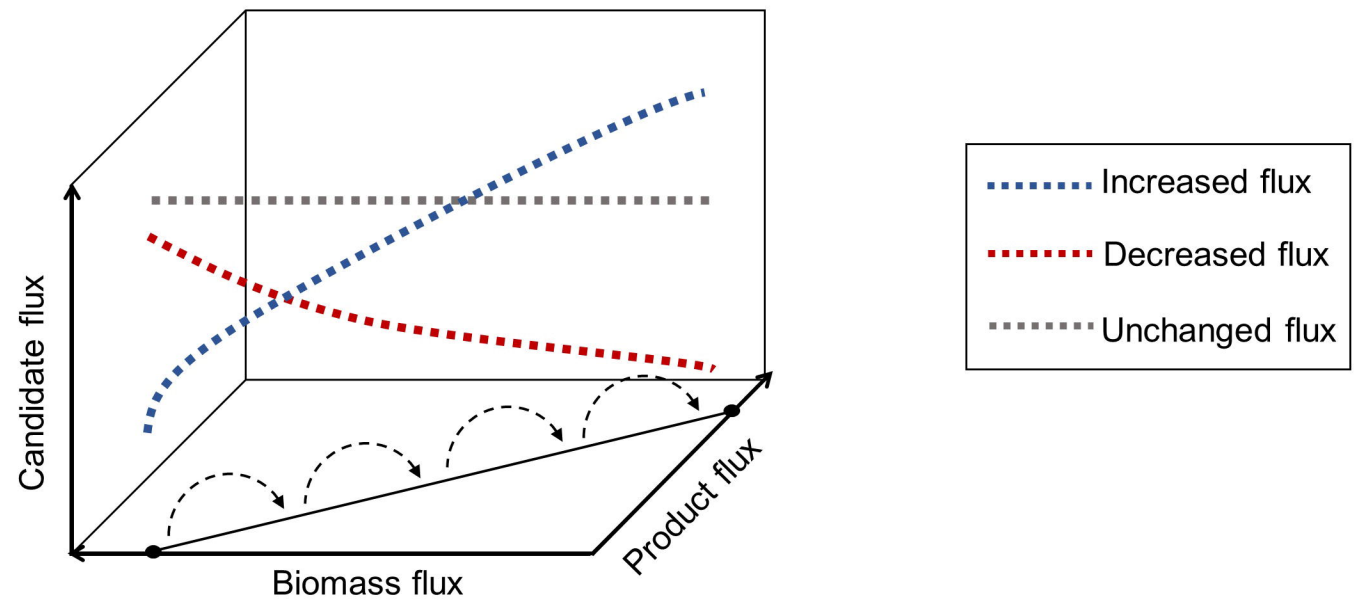


Constrained optimization

(b) Flux Scanning through Enforced Objective Flux (FSEOF)

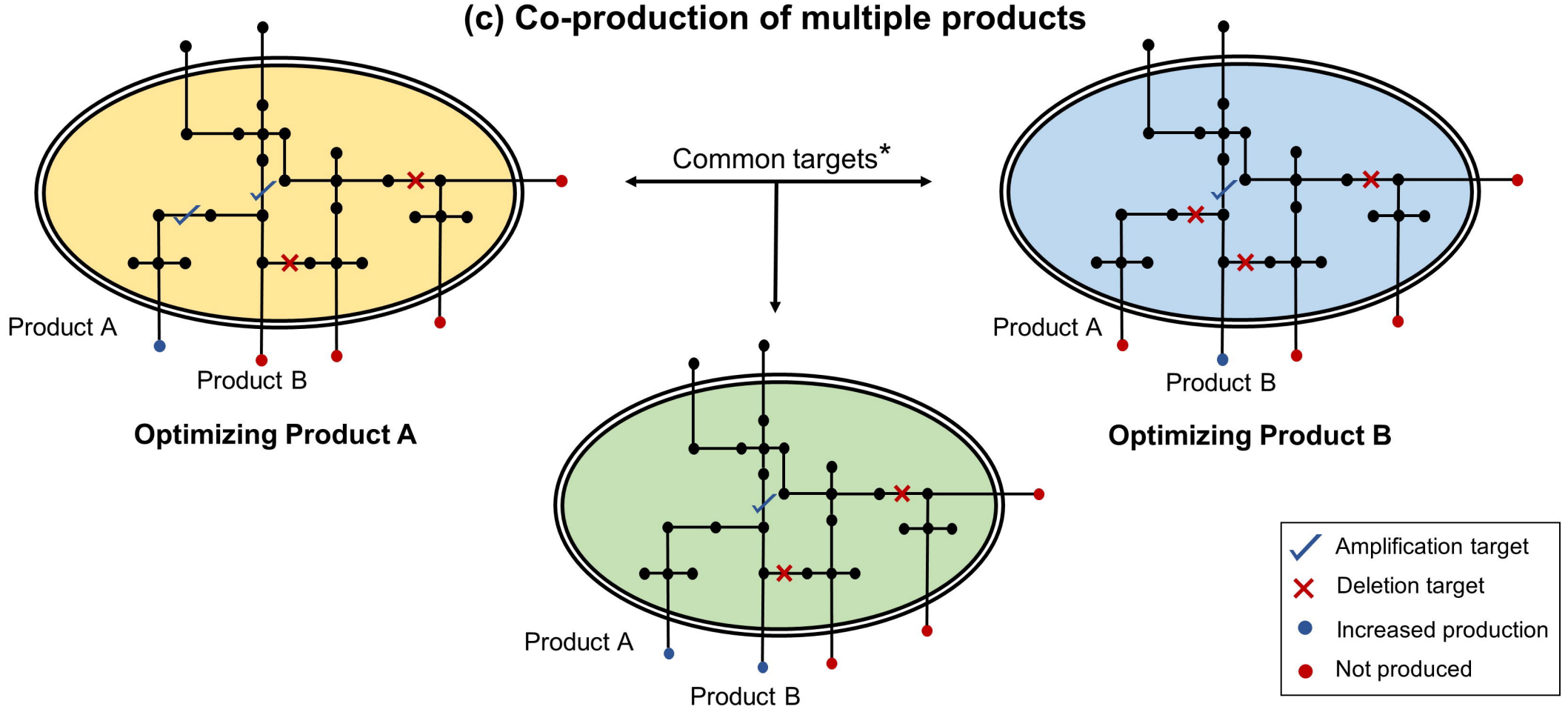


Enforcing flux towards product



Change in flux through the network

(c) Co-production of multiple products



Optimizing Product A

Optimizing Product B

Co-optimizing Product A and B