Metabolic engineering of *Escherichia coli* for biotechnological chondroitin production

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Background

Chondroitin is a glycosaminoglycan that is usually extracted from animal cartilage. It has been widely used in dietary supplements for osteoarthritis due to its anti-inflammatory properties. Microbial fermentation has been evaluated as a promising, safer and more sustainable source for this compound. The most studied microorganism for biotechnological production of chondroitin is the pathogen *Escherichia coli* O5:K4:H4 [1–5], since it naturally produces fructosylated chondroitin as a capsule constituent. Despite the advances on strain and bioprocess enhancement using *E. coli* K4 or recombinant strains, the yields obtained in microbial fermentations are not sufficient to meet the increasing demand. *In silico* analysis can provide insights on the design of strains with improved metabolic flux towards chondroitin production.

Methods

In the present work, a biosynthetic pathway for chondroitin production has been designed for a non-pathogenic *Escherichia coli* strain. The biosynthetic pathway is composed by two heterologous genes, coding for uridine diphosphate-glucose 4-epimerase and chondroitin synthase [6]. Using a stoichiometric genome-scale model of *E. coli* BL21 (iB21_1397) harboring the biosynthetic pathway, an optimization algorithm was applied to predict which gene modifications could result in improved chondroitin yields. The algorithm combined two objectives for evolutionary optimization, Biomass-Product Coupled Yield (BPCY) and Weighted Yield (WYield). Optflux software [7] was then used for phenotype simulation using parsimonious Flux Balance Analysis (pFBA) to predict the biomass and chondroitin yields for the most promising solutions.

Results

The *in silico* optimization of the designed *E. coli* metabolic model resulted in 76 solutions with improved chondroitin. These solutions comprised the modification of expression on a total of 39 different genes (**Figure 1**). The solutions were analyzed taking into consideration the frequency of each gene modification in the total solutions set and the correspondent value of expression.

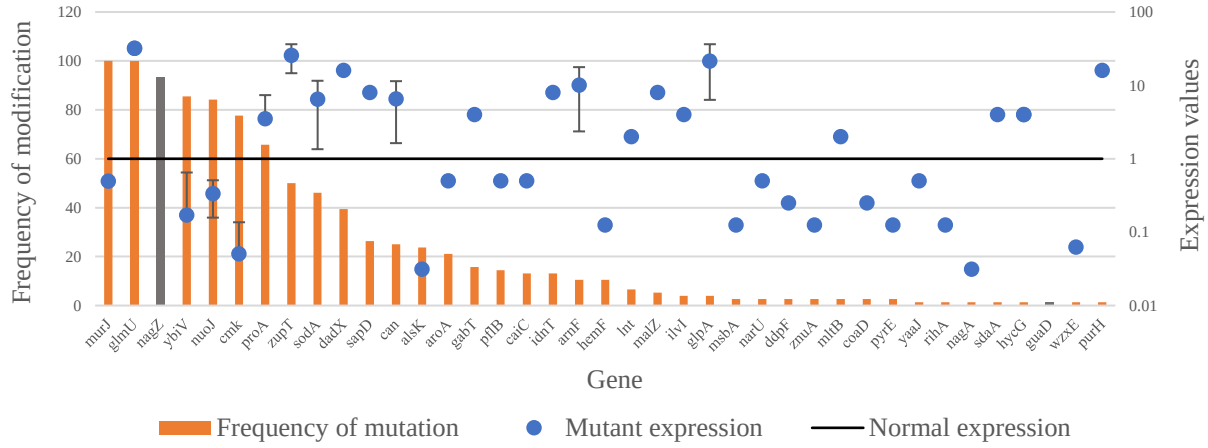

Figure 1. Frequency of gene modifications in the obtained 76 solutions and value of gene expression for each gene. Grey bars of *nagZ* and *guaD* genes correspond to knock-outs, where expression values are 0.

The number of modifications per solution varied between 8 to 10 genes. All solutions comprised *glmU* overexpression and *murJ* underexpression. GlmU encodes a bifunctional protein with both glucosamine-1-phosphate *N*-acetyltransferase and *N*-acetylglucosamine-1-phosphate uridyltransferase activities responsible for the synthesis of one of the precursors for the biosynthetic production of chondroitin, uridine diphosphate acetylglucosamine. MurJ encodes for lipid II flippase, which is involved in peptidoglycan synthesis. Also, 93% of the solutions included the knock-out of *nagZ*, encoding a β-hexosaminidase involved in peptidoglycan recycling. Peptidoglycan synthesis and recycling pathways are part of cell wall biogenesis, which is the known main competing pathway for chondroitin production since it uses common intermediates. Despite not predicted in any solution, the expression of uridine diphosphate-glucose dehygrogenase gene, *ugd*, is frequently identified on the literature as a limiting step for glycosaminoglycans production [8] and thus its overexpression is usually necessary to achieve interesting yields.
The solutions that showed better performance in the simulation in terms of BPCY are described in **Table 1**. The phenotype for these mutants was simulated using Optflux and the predicted yields on biomass and chondroitin are shown.

Table 1. Solutions from evolutionary optimization of *Escherichia coli* BL21 model with highest Biomass-Product Coupled Yield (BPCY) with the corresponding Weighted Yield (WYield), genes modified according to the type of expression modification, and the estimated biomass and chondroitin yields, as calculated using Optflux.

BPCY	WYield	Knock-Out	Underexpression	Overexpression	Biomass (mol g_{cells}^{-1} h^{-1})	Chondroitin (mol g_{cells}^{-1} h^{-1})
1.41649	2.91027	*nagZ*	*ybiV, alsK, aroA, pflB, murJ, narU*	*sodA, glmU, mltB*	0.30401	2.90794
1.41649	2.91027	*nagZ*	*ybiV, aroA, pflB, murJ, znuA*	*sodA, lnt, glmU, purH*	-	-

In the case of the second solution, no value of biomass or chondroitin yield could be estimated, probably because the values were too low to be handled by the Optflux software. Therefore, the first solution seems to be the most promising to produce chondroitin.

From the predicted modifications, only *glmU* overexpression has been performed in other studies in attempts to improve the production of a different glycosaminoglycan, hyaluronic acid, in recombinant microorganisms [9–12]. *In silico* predictions can provide less obvious mutants, accounting with the entire metabolic network present in the genome-scale metabolic model.

Based on the *in silico* results, the most promising gene modifications are being constructed in *E. coli* engineered with the chondroitin biosynthetic pathway. The *sodA*, *glmU* and *mltB* genes were amplified from the genome (**Figure 2**) and they will be cloned and expressed in plasmids. The *nagZ* deletion is being implemented using a Clustered Regularly Interspaced Short Palindromic Repeats(CRISPR)-Cas9 strategy. The underexpressions will be achieved though CRISPR interference (CRISPRi). With the construction of these mutants, the *in silico* results will be further validated.

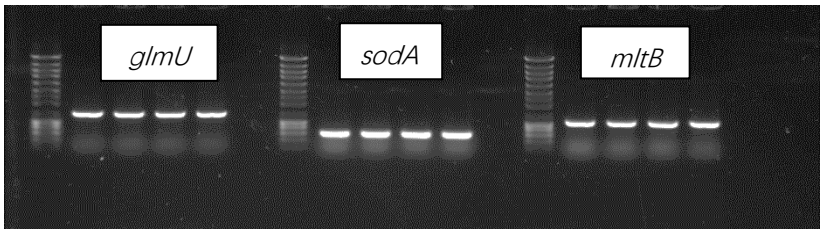

Figure 2. Gene amplification for cloning and expression in *Escherichia coli* strains engineered with chondroitin biosynthetic pathway.

Conclusion

The *in silico* prediction of *E. coli* mutants for chondroitin production can provide novel and more efficient strains to replace the use of animal tissues as chondroitin source. The predicted mutations will be further validated *in vivo*.

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