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Application of metabolic engineering to improve both the production and use of biotech indigo

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A fermentation process was developed for production of indigo from glucose using recombinant *Escherichia coli.* This was achieved by modifying the tryptophan pathway to cause high-level indole production and adding the *Pseudomonas putida* genes encoding naphthalene dioxygenase (NDO). In comparison to a tryptophan-overproducing strain, the first indigo-producing strain made less than half of the expected amount of indigo. Severe inactivation of the first enzyme of aromatic biosynthesis, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase (the *aroG*^{fbr} gene product), was observed in cells collected from indigo fermentations. Subsequent *in vitro* experiments revealed that DAHP synthase was inactivated by exposure to the spontaneous chemical conversion of indoxyl to indigo. Indigo production was thereafter improved by increasing the gene dosage of *aroG*^{fbr} or by increasing substrate availability to DAHP synthase *in vivo* by either amplifying the *tktA* (transketolase) gene or inactivating both isozymes of pyruvate kinase. By combining all three strategies for enhancing DAHP formation in the cell, a 60% increase in indigo production was achieved. Metabolic engineering was then further applied to eliminate a byproduct of the spontaneous conversion of indoxyl to indigo, thereby solving a serious problem with the use of bio-indigo in the final denim dyeing application.
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Introduction

The application of metabolic engineering to the production of commodity products is now a main focus of industrial biotechnology. Several examples of efforts to improve production of various compounds using metabolic engineering were collected in a recently published volume [20]. Lynd *et al* [22] also addressed the importance of metabolic engineering in the broader context of "biocommodity engineering."

At present, the scope of most metabolic engineering efforts is generally limited to the genetic manipulation of an organism to improve production of the desired compound. Indeed, this fits the original definition of metabolic engineering [2]. In addition to the modification of the primary pathway leading to the product, strain improvement through metabolic engineering may include, for example, increasing precursor supply [18] or preventing the synthesis of unwanted metabolites that are detrimental to the growth or productivity of the strain [1]. Moreover, metabolic flux analysis [12] may be used in the development of improved production strains. What is not as well recognized is that metabolic engineering can also be applied to problems beyond product yield. In this article, we describe our work to genetically engineer *Escherichia coli* for cost-competitive production of indigo by fermentation. The commercial and environmental justification for developing such a process was reviewed by Frost and Draths [16] and Frost and Lievense [17]. In addition, we show that metabolic engineering could be successfully applied to solve a problem far downstream of the bioreactor — in the ultimate denim dyeing application. Preliminary accounts of some parts of this work have been reported elsewhere [6,7,11].

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Materials and methods

Strains and plasmids

E. coli strain JB102 (F^- *trpR tnaA2* Δ*lacU169 serA*) is a derivative of PB103 [15] that also contains undefined mutations that are beneficial to tryptophan production [4]. *E. coli* strain FM5, a prototrophic derivative of *E. coli* K-12 [9], was the host for the construction of all indigo-producing strains. The double pyruvate kinase (*pykA pykF*) mutants of FM5 were constructed by insertional inactivation using the procedure described by Gosset *et al* [18]. The plasmids used in this work and their relevant descriptions are summarized in Table 1. Plasmids were maintained in the cells by addition of the appropriate antibiotic(s) to the culture medium. All antibiotics were used at a final concentration of 50 μg/ml.

Fermentation conditions

All indigo fermentations were performed in fed-batch mode in 14-l bioreactors (initial volume 6 l). The medium contained (per liter): 7.5 g of KH_2PO4, 2.0 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of citric acid, 7.0 g of amisoy (Traders Protein, Memphis, TN), 1.22 g of 98% H_2SO_4, 0.33 g of ferric ammonium citrate, and 5.0 g of glucose. A feed of 60% glucose was initiated upon exhaustion of the initial 5 g/l glucose in the batch phase. The glucose feeding profile consisted of an exponential feeding period (0.62–1.39 g/h over a period of 8.5 h) followed by a constant feed of 1.39 g/h. Tryptophan fermentations were performed similarly, using a medium identical to the indigo fermentations but without amisoy. For bioconversion experiments (tryptophan to indigo), strain FM5/Fd911 was grown in fed-batch

Table 1 Plasmids used in this work

Plasmid	Vector backbone [reference]	Relevant promoter-cloned gene(s)	Purpose/comments [source or reference]
pBE7	pACYC184 [10]	P_{lacUV5}-*aroG*^{fbr}, P_{lacUV5}-*trpE*^{fbr}*DCBA*	Tryptophan production plasmid [[4]; this study]
pBMW	pBR322 [8]	P_{lacUV5}-*aroG*^{fbr}, P_{lacUV5}-*trpE*^{fbr}*DCB*/*26A*	Indole production plasmid [this study]
Fd911	pAC1 [23]	λP_L-NDO	NDO expression plasmid [23]
pTacFd911	pAC1	P_{tac}-NDO	NDO expression plasmid [this study]
pCL-*aroG*^{fbr}	pCL1920 [21]	P_{tac}-*aroG*^{fbr}	Amplification of DAHP synthase activity [this study]
pCL-*tktA*	pCL1920	*tktA* (native promoter)	Amplification of transketolase activity [18]
pCL-*tktA*-*aroG*^{fbr}	pCL1920	*tktA* (native promoter), P_{tac}-*aroG*^{fbr}	Combined amplification of DAHP synthase and transketolase activities [this study]
pCL-IHA	pCL1920	P_{tac}-*iha*	Introduction of isatin hydrolase activity [[26]; this study]

mode as described above for indigo fermentations, and a feed of tryptophan (400 g/l in NaOH) was initiated 14 h after the start of the glucose feed. The initial tryptophan feed rate was 0.2 g/min and was increased to 0.5 g/min over 3 h.

Assays and other procedures

Indigo was assayed by mixing appropriate dilutions of whole fermentation broth (i.e., cells and medium) with either dimethylsulfoxide or dimethylformamide to solubilize the indigo, centrifuging the mixture to remove the biomass, and reading the absorbance of the supernatant at 620 nm. Indigo concentrations were calculated by comparison to a standard curve constructed using synthetic indigo. All other compounds of interest were measured by high-performance liquid chromatography (HPLC). 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase was assayed as described previously [5]. Free indoxyl for fermentor feeds and for *in vitro* DAHP synthase inactivation assays was prepared as follows: a 30% suspension of indoxyl acetate (Sigma St. Louis, MO, cat. no. I-3500) was enzymatically hydrolyzed using Lumafast lipase (Genencor International, Palo Alto) in the presence of K_2HPO_4 (to maintain alkaline pH during hydrolysis). The resultant indoxyl slurry was rapidly washed with 10 vol of ice-cold water and indoxyl was resuspended in water to a 30% slurry. To prevent oxidation, the slurry was kept under nitrogen during feeding or kept frozen at $-20^{\circ}C$ for storage. Experiments to evaluate the *in vitro* inactivation of DAHP synthase were carried out as follows: extract from *E. coli* strain AB3248/pRW5 [18,25] overexpressing AroG^{fbr} was added to assay buffer (100 mM potassium phosphate buffer, pH 7.0, 1 mg/ml BSA, and 100 U of catalase; Sigma C-30) to give a total volume of 100 μl per reaction. Inactivation was started by addition of the indicated concentration of indoxyl and incubation was continued at 20°C in the presence of air. At specified time points, appropriate reaction replicates were withdrawn with a 12-channel pipettor and desalted over home-fabricated spin columns, pre-equilibrated with DAHP synthase reaction buffer, and set up in 96-well microtiter plate format to recover enzyme separated from inhibitor. Remaining enzyme activity was determined by the colorimetric DAHP synthase assay [5]. All data were fitted to a first-order decay rate equation.

Results and discussion

Construction of a strain for high-level production of indigo from glucose by fermentation

Ensley *et al* [14] first reported production of indigo in recombinant *E. coli* cells carrying the cloned *Pseudomonas putida* genes encoding the four subunits of naphthalene dioxygenase (NDO). The basis for indigo production (Figure 1) was the combined activities of the native *E. coli* enzyme tryptophanase (converted tryptophan in the culture medium to indole) and the cloned *P. putida* NDO (converted indole to indigo). Although an industrial process for bioconversion of tryptophan [13] or indole to indigo using *E. coli* expressing NDO is feasible, it is not economically attractive. Indole conversion is further complicated by the toxicity of indole to *E. coli* [3]. Clearly, a metabolic engineering strategy coupling *de novo* synthesis of indole with NDO activity in a single organism is the most effective approach. Murdock *et al* [23] reported the construction of a modified *E. coli* tryptophan operon containing a mutation in the *trpB* gene that inactivated the terminal step in tryptophan biosynthesis (conversion of indole+serine to tryptophan by the β-subunit of tryptophan synthase). This resulted in accumulation of indole in the cultures of *E. coli* expressing the modified *trp* operon. By adding cloned *P. putida* NDO to the strain, *de novo* indigo production was achieved [23]. Although the level of indigo produced was not industrially relevant, it provided the basis for further metabolic engineering of the organism to increase indigo production.

Independently, a recombinant strain of *E. coli* producing high levels of tryptophan was developed [6]. By merging this tryptophan technology with NDO, the first *E. coli* strain capable of high-level indigo production directly from glucose by fermentation was created. The base *E. coli* production strain used in the present work is designated FM5/pBMW, pTacFd911. The salient features of this strain are as follows (refer also to Table 1 and Figure 1). Plasmid pBMW contains the *E. coli aroG*^{fbr} gene (encodes a feedback-resistant DAHP synthase [5]) and the modified *trp* operon carrying the *trpB26* mutation [23]. The *trpE* gene in the modified operon was also mutated to render its product, anthranilate synthase, insensitive to feedback inhibition by tryptophan [6]. The *aroG*^{fbr} gene and the *trpB26* operon were cloned under control of tandem *lacUV5* promoters. Thus, plasmid pBMW carries the genes required for high-level production of indole. Plasmid Fd911 contains the *P. putida* genes coding for NDO cloned under control of the λP_L promoter [23]. Plasmid pTacFd911 is identical to plasmid Fd911, except that the NDO genes were cloned under control of the *tac* promoter.

Discrepancy between tryptophan production and indigo production

Figure 2A shows a representative bioconversion of tryptophan to indigo by *E. coli* strain FM5/Fd911 [23] expressing only the native, chromosomally encoded tryptophanase and the cloned *P. putida* NDO genes. In these bioconversions, tryptophan was fed to the fermentor simultaneously with glucose, which was maintained at growth-limiting levels (i.e., fed-batch mode). Tryptophan was

efficiently converted to indigo in the fermentor, producing 23 g/l in 42 h. The overall yield of indigo from the fed tryptophan was about 95%.

Figure 2B shows a representative tryptophan fermentation using *E. coli* strain, JB102/pBE7. Plasmid pBE7 contains the *aroG*^{fbr} gene and the *trp* operon (wild type except for the presence of the feedback-resistant *trpE* gene). Under these conditions, tryptophan production reached >40 g/l in 50 h. For the last half of the fermentation, tryptophan was produced at the theoretical maximum yield of 22% [24] from glucose.

Based on the results shown in Figure 2A and B, by modifying the tryptophan operon to produce indole, and combining these genes with NDO in a single organism, a fermentation process that produces indigo at a molar concentration equivalent to tryptophan production in strain JB102/pBE7 should be possible. However, this was not the case. The dashed line in Figure 2B shows the expected indigo production in a single strain (e.g., FM5/pBMW, pTacFd911) where *de novo* indole production has been combined with cloned NDO (2 mol of tryptophan are required to produce 1 mol of indigo). The actual indigo production in FM5/pBMW, pTacFd911 is shown by the bottom curve in Figure 2B. Indigo was produced at less than half of the expected amount.

Although the unrestricted growth rate of FM5/pBMW, pTacFd911 is lower than FM5/Fd911 or JB102/pBE7, in the fed-batch fermentations used here, the growth rate was restricted (by the glucose feeding rate) at a rate similar to that used for the tryptophan fermentation or the tryptophan-to-indigo bioconversion. Thus, poor growth did not account for the discrepancy between the expected and actual indigo production (this was confirmed by measurement of the biomass). However, there were several other possible explanations for the lower-than-expected indigo production by the strain. For example, the genetic load of expressing >10 cloned genes could have affected production, or product toxicity could have been a factor. But a simpler explanation was that there was a rate-limiting reaction(s) in the overall biosynthetic pathway leading to indigo. Culture supernatants from the bioreactors were examined by HPLC for accumulation of several of the pathway intermediates between DAHP and indole (dehydroshikimate, shikimate, 5-enolpyruvylshikimate 3-phosphate, and anthranilate). However, no accumulation of these intermediates was detected in the fermentation broth (data not shown), suggesting that the enzymatic reactions in this segment of the pathway were not responsible for the low indigo production.

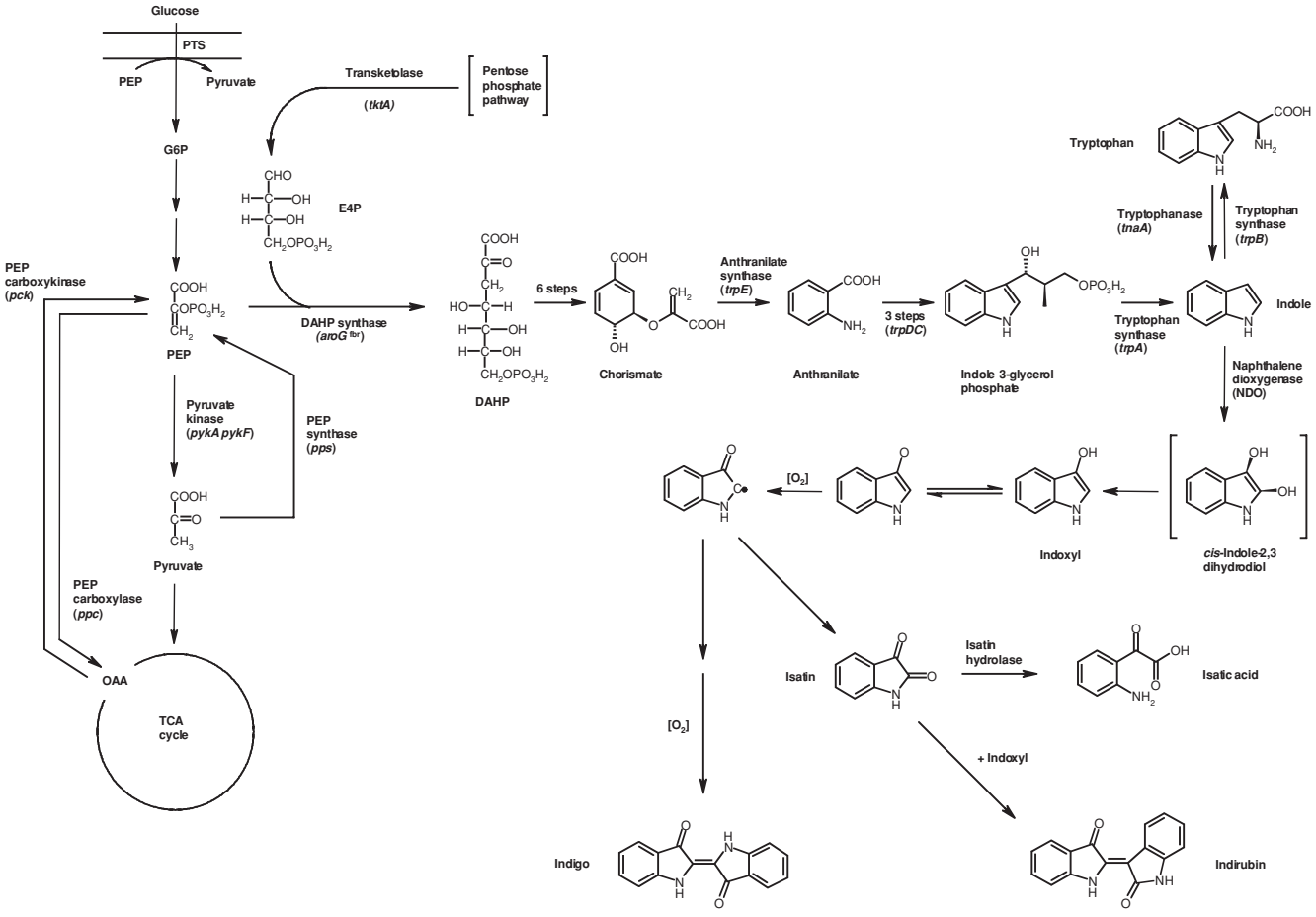

Figure 1 Indigo biosynthetic pathway created by the merger of indole biosynthesis and NDO activity in one organism. The central metabolic pathways that produce the precursors of aromatic biosynthesis (PEP and E4P) are also represented in simplified form to show the connection of transketolase and pyruvate kinase to DAHP production *via* DAHP synthase. Also indicated are the conversion of tryptophan (produced *de novo* or supplied exogenously) to indole *via* tryptophanase and the activity of isatin hydrolase (see text). The *cis*-indole-2,3-dihydrodiol is presumed to be the final enzymatically formed intermediate; all subsequent reactions are spontaneous. It has not been determined whether the diol or indoxyl is exported from the cell, but the oxidation reactions from indoxyl to indigo presumably must occur extracellularly. All indigo is extracellular.

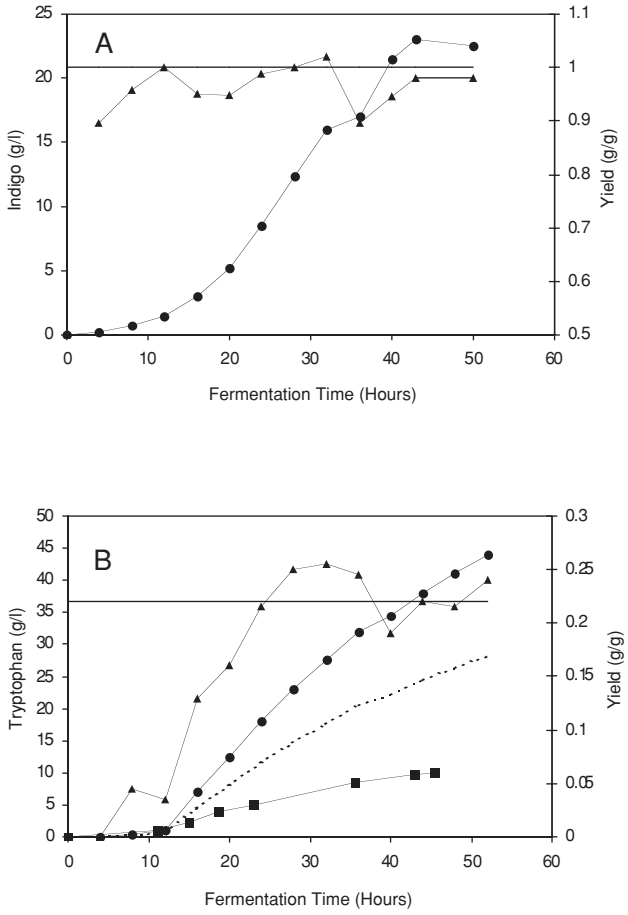

Figure 2 (A) Bioconversion of tryptophan to indigo by *E. coli* strain FM5/Fd911 expressing native (chromosomally encoded) tryptophanase and cloned *P. putida* NDO. (●) Indigo production. (▲) Yield (gram of indigo produced per gram of tryptophan fed). The solid line represents the theoretical maximum yield for the conversion (100%). (B) Tryptophan and indigo production by recombinant *E. coli* strains. (●) Tryptophan production by strain JB102/pBE7. (▲) Tryptophan yield (gram of tryptophan produced per gram of glucose consumed). (----) Expected indigo production for strain FM5/pBMW, pTacFd911 based on tryptophan production by strain JB102/pBE7. (■) Actual indigo production by *E. coli* strain FM5/pBMW, pTacFd911. The solid line represents the theoretical maximum yield of tryptophan from glucose (22% [24]).

The first indication of the basis for the difference in tryptophan and indigo production came from intermediate feeding studies. Anthranilate (an intermediate in tryptophan biosynthesis; see Figure 1) was fed to indigo fermentations using strain FM5/pBMW, pTacFd911, pCL-IHA to determine if rate-limiting steps existed between anthranilate and indigo (plasmid pCL-IHA contains the cloned isatin hydrolase gene, as described below). Numerous experiments were attempted before a suitable anthranilate feeding rate was determined, as feeding of anthranilate at even a moderate rate was found to cause a rapid loss of metabolic activity of the cells. However, once an acceptable feed rate was determined, the addition of anthranilate indeed increased indigo production significantly (1.6-fold at 48 h fermentation time; data not shown). This result suggested that the unexpectedly low indigo production by FM5/pBMW, pTacFd911 was not due to reactions in the latter half of the overall pathway.

Based on this result and on the fact that we did not detect accumulation of intermediates between DAHP and anthranilate in the culture medium, we turned our attention to the first enzyme of the aromatic amino acid pathway, DAHP synthase. As mentioned above, the cloned DAHP synthase gene in the indigo production strains is a mutant form of *aroG* that encodes a DAHP synthase where the normal allosteric inhibition by phenylalanine has been removed. In previous work, we had determined that the DAHP synthase activity in JB102/pBE7 cells harvested across a tryptophan fermentation was high, and remained so throughout the fermentation. However, when we performed this analysis on cells taken from indigo fermentations, this was not the case. Figure 3 shows tryptophan production and indigo production in fermentations using strains JB102/pBE7 and FM5/pBMW, pTacFd911, pCL-IHA, respectively, and the DAHP synthase activity in crude extracts of cells removed at various time points during the fermentations. As observed previously, the DAHP synthase activity in JB102/pBE7 remained at a high level across the whole tryptophan fermentation. In contrast, DAHP synthase activity in FM5/pBMW, pTacFd911, pCL-IHA started out at a high level and then dropped steadily to less than 20% of the activity measured in cells withdrawn from the fermentor before any indigo production was detected. This result was observed in every subsequent indigo fermentation. In a related set of experiments (not shown), indoxyl was added to fermentations using a strain of *E. coli* overexpressing just the cloned $aroG^{fbr}$ gene, and the response of DAHP synthase activity in the cells to the extracellular (spontaneous chemical) indigo production in the fermentor was measured. Within minutes after indoxyl addition, a sharp decrease in DAHP synthase activity was observed.

Further metabolic engineering to improve indigo production

Our results suggested that one or more of the spontaneous extracellular chemical reactions in the transformation of indoxyl to indigo was responsible for the loss of DAHP synthase activity inside the cells. This possibility was further investigated in *in vitro* experiments. Figure 4 shows the inactivation of DAHP synthase by indoxyl in a concentration-dependent manner. Inactivation was dependent on the presence of oxygen and took place only during the

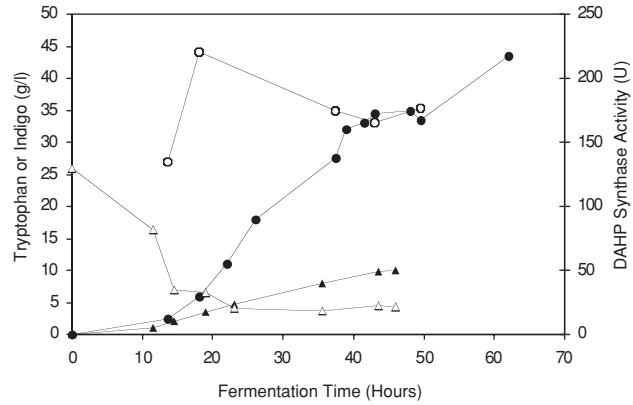

Figure 3 Tryptophan and indigo production by *E. coli* strains JB102/pBE7 and FM5/pBMW, pTacFd911, pCL-IHA respectively, and DAHP synthase specific activity in cells taken from the fermentors at various time points. (●) Tryptophan production by JB102/pBE7. (○) DAHP synthase activity in JB102/pBE7. (▲) Indigo production by FM5/pBMW, pTacFd911, pCL-IHA. (△) DAHP synthase activity in FM5/pBMW, pTacFd911, pCL-IHA.

active conversion of indoxyl to indigo, suggesting that an intermediate or a byproduct of this chemistry was the inactivator. Involvement of the active site was suggested by the finding that preincubation of the enzyme with one of its substrates, phosphoenolpyruvate (PEP), provided partial protection against inactivation (data not shown). Protection was not observed when the enzyme was preincubated with its other cosubstrate, erythrose 4-phosphate (E4P).

These results suggested that if more PEP were available to DAHP synthase in the cell, indigo production could be improved because the enzyme might be protected against inactivation. In related work [18], we showed that the commitment of carbon from central metabolism to the aromatic amino acid pathway could be increased by manipulating genes responsible for the production or consumption of the precursors of aromatic biosynthesis, E4P and PEP. Two of the ways this was achieved were to amplify the *tktA* gene, encoding transketolase, and inactivate one or both isozymes of pyruvate kinase (PK, encoded by the *pykA* and *pykF* genes) [18]. The basis for these strategies is illustrated in Figure 1. *A priori*, it seems that amplification of *tktA* would increase E4P availability while inactivation of PK would increase PEP availability. However, although both approaches work in terms of increasing carbon flow to aromatic biosynthesis, the basis for the improvement has not been established [4]. Thus, we tested both approaches for the ability to improve indigo production. In addition, we simply increased the dosage of the cloned *aroG*^{fbr} gene (by adding plasmid pCL-*aroG*^{fbr}) to see what, if any, effect this might have on indigo production. Plasmids used to construct the strains for these experiments are described in Table 1.

Figure 5 shows that all three individual approaches (increased dosage of *aroG*^{fbr} or *tktA*, or inactivation of *pykA*/*pykF*) increased indigo production by approximately 30%. There was no synergistic effect of amplified *tktA* and the *pyk* mutations without the increased dosage of *aroG*^{fbr}. However, when all three strategies were combined in one strain (FM5 *pykApykF*/pBMW, pTacFd911, pCL-*tktA*-*aroG*^{fbr}), the improvement in indigo production was doubled compared to any of the individual strategies. At approximately 60 h of fermentation time, the indigo production in strain FM5 *pykApykF*/pBMW, pTacFd911, pCL-*tktA*-*aroG*^{fbr} was 60% higher than the base control strain, FM5/pBMW, pTacFd911. Moreover, indigo production by strain FM5 *pykApykF*/pBMW, pTacFd911, pCL-*tktA*-*aroG*^{fbr} reached >18 g/l when the cultivation was allowed to continue for an additional 12 h.

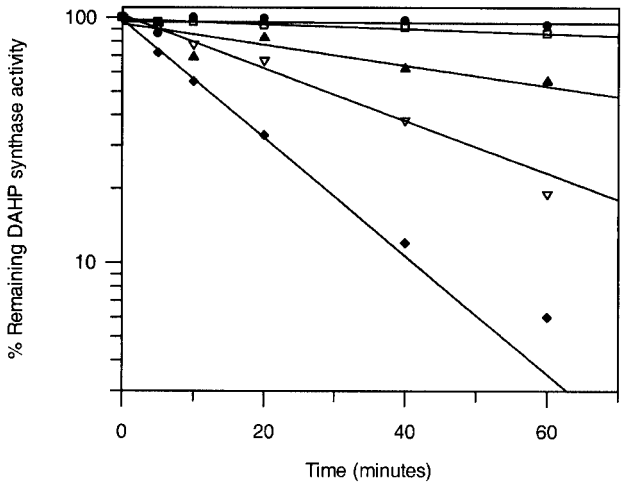

Figure 4 Inactivation of DAHP synthase (AroG^{fbr}) as a function of the initial indoxyl concentration. Details of the assay procedure are given in Materials and Methods. Initial indoxyl concentrations are indicated as follows: (●) no indoxyl; (□) 0.313 mM; (▲) 0.625 mM; (▽) 1.25 mM; (◆) 2.5 mM.

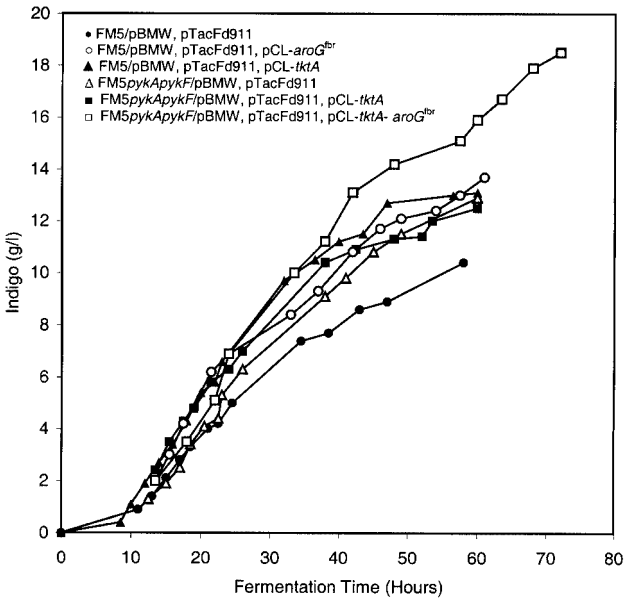

Figure 5 Effect of manipulating DAHP synthase activity and/or precursor supply on indigo production. For convenience, the strains and symbols are shown directly on the figure. Refer to Table 1 and Materials and Methods for descriptions of the host strains and plasmids.

These results strongly suggest that indigo production in the control strain FM5/pBMW, pTacFd911 is limited by the first step in aromatic biosynthesis (conversion of PEP and E4P to DAHP). However, the underlying basis for this limitation in DAHP formation cannot be determined from our results. It is possible that even at the lowest ("inactivated") level of DAHP synthase activity in the cells, indigo production is still not limited by DAHP synthase activity, but rather by availability of PEP and/or E4P. Further increasing DAHP synthase activity (by adding plasmid pCL-*aroG*^{fbr}) would be expected to pull more substrates into aromatic biosynthesis, translating to improved indigo production. Substrate limitation in the control strain FM5/pBMW, pTacFd911 could also explain why amplification of transketolase or inactivation of pyruvate kinase improved indigo production even without further increasing DAHP synthase activity. However, in light of the protective effect of PEP against inactivation of DAHP synthase *in vitro*, it cannot be ruled out that the *in vivo* activity of DAHP synthase is enhanced in strains having amplified transketolase or inactivated pyruvate kinase. Regardless, indigo production was greatly improved by these approaches, and the results illustrate the power of metabolic engineering to overcome unique problems in bioprocesses.

Use of metabolic engineering to overcome a problem in the final product application

Indigo produced by fermentation (*de novo* from glucose or by bioconversion of tryptophan) was tested in large-scale denim

Table 2 Indirubin content in biosynthetic and chemical indigo (20% pastes) and in denim dyed with these products

Sample	Cloned isatin hydrolase gene in production strain	Indirubin level in indigo product (mg of indirubin/ml of 20% indigo paste)	Indirubin concentration in dyed denim (mg/g of denim)
Bio-indigo	–	0.91	0.061
Bio-indigo	+	0.46	0.011
Chemical indigo	N/A	0.03	below detectable limit

dyeing processes. Finished denim garments produced using this indigo showed an undesirable red cast. This was attributable to the presence of indirubin, a structural isomer of indigo (Figure 1) in the biologically produced indigo [11]. Indirubin is formed by combination of indoxyl and isatin, a trace byproduct in the spontaneous oxidation reactions converting indoxyl to indigo. Indirubin is not unique to biological indigo; it is also formed during the chemical synthetic process used for indigo manufacture. However, most of the indirubin is removed during the chemical manufacturing process and is present at a very low level in the finished product.

We found that the level of isatin in the fermentor, and consequently the level of indirubin in the finished indigo product, could be decreased to some extent through modification of the fermentation conditions. However, a genetic solution to the problem was also pursued. Isatin was reported to be an intermediate in the biodegradation of indole [19]. Based on this information, Weyler *et al* [26] isolated the indole-degrading bacterium, *P. putida* strain WW2, and from it cloned and sequenced a gene encoding the enzyme isatin hydrolase [converts isatin to isatic acid (see Figure 1), which is not a potential precursor of indirubin]. This gene was then used to create new indigo-producing strains that contained (in addition to all the activities required for indigo production) a high level of isatin hydrolase activity. The presence of isatin hydrolase activity in the cells did not improve indigo production in the fermentor, but it did have the desired effect of decreasing the indirubin content of the indigo recovered. Table 2 shows that having the cloned isatin hydrolase in the production organism resulted in a 50% and 80% decrease in the indirubin content of the final indigo product (20% paste) and the dyed denim, respectively (the reason for the even lower indirubin content in the dyed denim is not known). The indirubin content in the pastes and the dyed denim produced with the biosynthetic indigo were still higher than that observed for the chemical indigo. Nevertheless, when the biological indigo produced using the isatin hydrolase-containing strain was tested in large-scale denim dyeing trials, its performance was equivalent to chemically produced indigo.

The work reported here illustrates that metabolic engineering can be applied not only to create production organisms with improved yields, but also to solve problems encountered in the production and even in the ultimate application of the product. In the case of biological indigo, metabolic engineering was applied to (1) create a hybrid pathway for *de novo* synthesis; (2) overcome a limitation in a key biosynthetic step; and (3) eliminate the precursor of a problematic byproduct that negatively affected the performance of the product relative to its synthetic counterpart.

The indigo-producing strains used in this work were prototypes. Likewise, the laboratory-scale fermentations used were designed for reliable testing of strains and were not highly optimized. Further improvements, leading to decreased process costs, could be achieved through genetic optimization and streamlining of the strain and through fermentation process development. In addition, higher indigo production levels could be achieved through identification and appropriate manipulation of the expression of other important genes.

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