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PAPER

Roles of sulfite oxidoreductase and sulfite reductase in improving desulfurization by *Rhodococcus erythropolis*

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Rhodococcus erythropolis has been widely studied for desulfurization. However, activity levels required for commercial application have not been achieved. A major limitation of the current work in biodesulfurization is inadequate information regarding sulfur metabolism generally, and in particular the metabolism of the sulfur obtained from dibenzothiophene (DBT) metabolism *via* the 4S pathway. In this work, we have investigated the possible routes taken by the sulfur from DBT to convert into biomass or other metabolites. We propose two alternate hypotheses. In the first, we hypothesize that the cell can convert *via* sulfite reductase (SR) the sulfite from the metabolism of DBT into sulfide that can be assimilated into biomass. However, in the process, it may convert any excess sulfite into extracellular sulfate *via* sulfite oxidoreductase (SOR) to avoid the toxic effects of sulfite. In the second, we speculate that the cell cannot assimilate the sulfite directly into biomass *via* SR. It must first use SOR to produce extracellular sulfate, and then recapture that sulfate into biomass *via* SR. Thus, either way, we propose that SOR and SR activities, in addition to *dsz* genes and cofactors, may be critical in increasing desulfurization levels significantly. In particular, we suggest that the simultaneous increase in SOR activity and decrease in SR activity can enable increased desulfurization activity.

Introduction

Several bacterial species metabolize polyaromatic sulfur heterocycles (PASHs) such as dibenzothiophene (DBT) and its derivatives commonly found in fossil fuels. Of them, *rhodococci* are widely studied for their ability to desulfurize DBT *via* a well-known non-destructive 4S pathway conferred by an operon of *dsz* genes.[1] This pathway involves four thermodynamically favorable reactions that convert DBT to sulfur-free 2-hydroxybiphenyl (HBP) with concomitant release of sulfite. It is of special interest, as it specifically cleaves the C–S bond in DBT without affecting the carbon skeleton and does not lead to any undesirable loss of calorific value. Moreover, it requires lower energy, temperatures, and pressures than hydro-desulfurization, the prevalent desulfurization method.[2]

Numerous works are available on the characterization of the desulfurizing *rhodococci* and their activities.[3–5] A desulfurization activity (DA) of nearly 1.2–3.0 mmol gdcw^{-1} h^{-1}[6–8] is desirable for a competitive commercial biodesulfurization process. However, the desulfurization activities of the wild type strains are too low for commercialization. Several studies exist on optimal conditions[9–11] and media compositions[12–16] for obtaining higher levels of desulfurization. However, sulfur metabolism in general, and the metabolism of sulfur derived from the desulfurization of DBT in particular are not well understood in *Rhodococcus*. For instance, the 4S pathway is known to produce sulfite, but it is unclear how the cell (1) incorporates sulfite into biomass, and (2) excretes sulfate.[17]

A major challenge is to enhance DA, as the activity of wild strains is too low for an industrial process.[26] Genetic engineering has been used to create strains with improved DA. These strains have involved alterations[18–21] to copy number of *dsz* gene(s), promoters expressing *dsz* genes, *dsz* operon, and genes that supply cofactors needed by Dsz enzymes and have achieved up to 25-fold increase in DA as compared to the parent strains. However, all the above strategies have focused almost exclusively on 4S pathway elements, and they have not achieved desired activity levels. Several investigators have demonstrated that different bacterial hosts containing identical *dsz* genes exhibit different DA, substrate range, and temperature ranges for the 4S pathway.[15,26] Clearly, there is a need to look beyond the 4S pathway.

Repression of DA in both the parent and genetically modified strains has been observed in the presence of sulfate. Therefore, some researchers[22,23] have designed recombinant

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strains using promoter deletion and replacement strategies to overcome sulfate repression. However, Franchi *et al.*[23] observed that the activity of the promoter-replaced strains was also reduced in the presence of sulfate by nearly 50%. Therefore, they postulated sulfate to exert additional regulatory effects on desulfurization.

Recently a genome-scale *in silico* metabolic model of *Rhodococcus erythropolis* was developed and shown to be useful in determining preferred carbon sources for optimum DA.[1] In this study, the metabolic model of *R. erythropolis* is expanded, specifically to better address sulfur metabolism and we present hypotheses regarding the incorporation of sulfur from DBT into biomass, and the key roles of SOR and SR in DA. Based on these insights, we propose strategies for improved desulfurization.

Results and discussion

The growth-related sulfur need of *R. erythropolis* is low (about 0.5–1% of dry cell weight), which naturally constrains its DA. Clearly, if we wish to achieve desulfurization rates beyond this natural limit, then we must either increase this nutritional requirement for sulfur by engineering the environment and/or the genome of *R. erythropolis*, or induce *R. erythropolis* to accumulate/excrete sulfur in some form.

The discussions by Kilbane and Le Borgne[17] suggest a clear need for thoroughly understanding the metabolism of sulfur in the 4S pathway. They make it clear that factors other than the *dsz* genes need to be considered, if optimum desulfurization activity is to be achieved. While the sulfur atom in DBT is oxidized to sulfite *via* the 4S pathway before getting assimilated into biomass,[6,24] the specific enzymes and pathways employed by *Rhodococcus* for the utilization of sulfite derived from DBT desulfurization are not well understood. A major inexplicable phenomenon in desulfurizing strains is the formation and excretion of sulfate when sulfite is the major sulfur metabolite obtained from DBT. Thus, a key issue that must be addressed in an improved metabolic model of *R. erythropolis* is the observation that sulfate is present extracellularly in desulfurization-competent cultures utilizing DBT as the sole source of sulfur.[3,25–27]

Sulfate formation in the 4S pathway

We propose two alternate plausible hypotheses for explaining the sulfate formation from sulfite during DBT desulfurization (Fig. 1). The first is based on the presence and toxicity of excess sulfite, and the second is based on enzyme compartmentalization.

(i) Toxicity of excess sulfite. Sulfite is toxic to microbial cells,[28,29] so the cells would naturally avoid its accumulation. However, being a proven intermediate metabolite in the 4S pathway, its accumulation inside *R. erythropolis*, even if temporary, is highly plausible. The direct incorporation of sulfite derived from DBT into biomass may occur; however, the rate of sulfite utilization may be less than the rate of sulfite formation by the 4S pathway resulting in the intracellular accumulation of toxic levels of sulfite.[28,29] Assuming that this happens, *R. erythropolis* must have a mechanism to limit the toxic effect of sulfite. Searching for such a mechanism, we learnt that prokaryotes can indeed oxidize sulfite[29–31] to sulfate *via* two pathways: direct and indirect. SOR catalyzes the former, and a reductase and a sulfurylase effect the latter. To our knowledge, the presence of SOR in *R. erythropolis* has

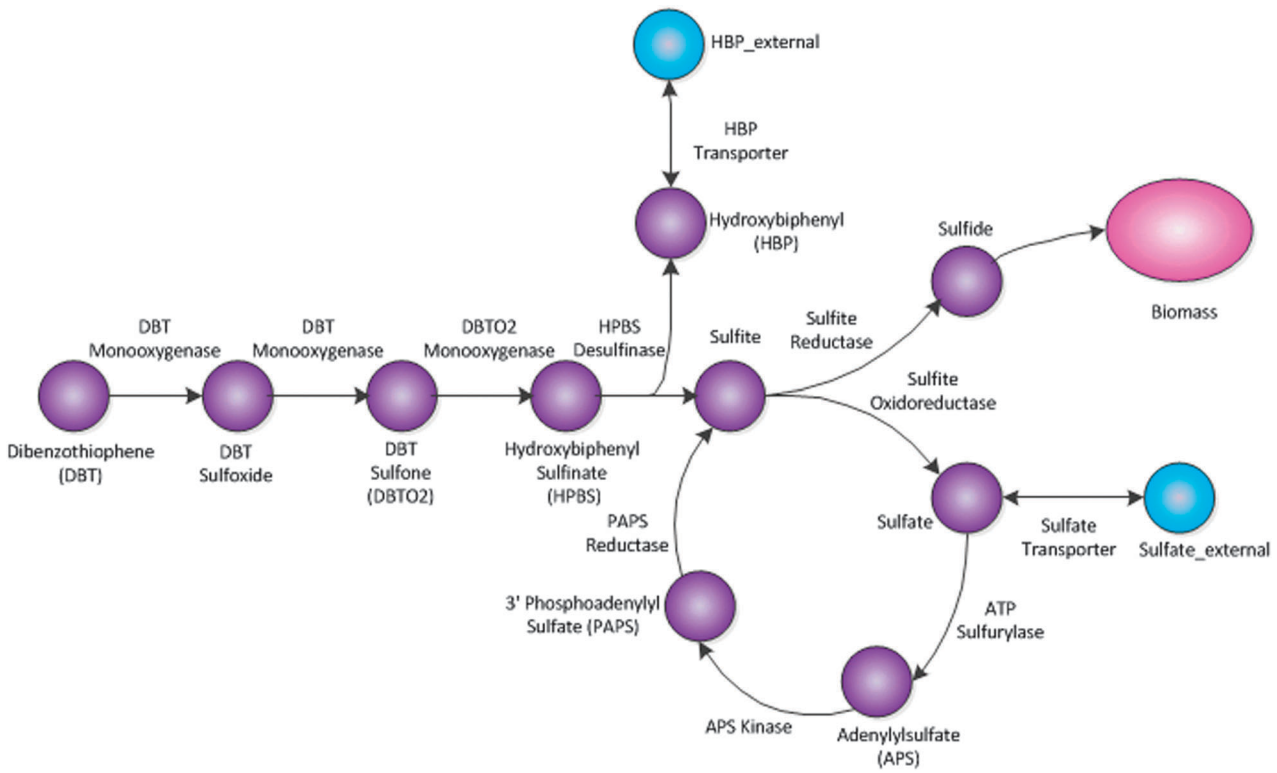


Fig. 1 Formation and consumption of sulfite and sulfate for biomass formation during DBT metabolism.

been neither hypothesized nor confirmed in the literature. However, our BLASTp analysis using the amino acid sequence of SOR (E.C. 1.8.2.1) located an ORF with the gene name and locus tag of RER_39830 in the genome of *R. erythropolis* PR4. This can encode for SOR that can convert toxic sulfite to non-toxic sulfate.

Having explained the formation of sulfate in *R. erythropolis*, we now elucidate sulfite accumulation, especially when *R. erythropolis* may have no incentive to produce sulfite beyond its growth-related needs. Consider scenarios in which sulfur is limiting for cell growth. For assimilation into biomass, perhaps sulfite is first converted to sulfide by sulfite reductase (SR) (EC 1.8.1.2). Van Der Ploeg *et al.*[32] reported that SR is encoded by the *cysJI* genes in *Bacillus subtilis*. They also observed that sulfate induces the expression of *cysJI*, and any deletion in *cysJI* blocks the utilization of sulfite or sulfate by the organism. Using the model of Aggarwal *et al.*,[33] we studied the role of SR in sulfur metabolism in *R. erythropolis*. For this, we inactivated the SR enzyme by restricting the flux through the associated reaction to be zero. Under this condition *R. erythropolis* was unable to exhibit any *in silico* growth when sulfite and sulfate were provided solely or in combination as sulfur source(s). This confirmed that SR is essential for utilizing sulfite or sulfate by *R. erythropolis*. We hypothesize that SR is probably not active in *R. erythropolis* initially in the absence of sulfate. Thus, the sulfite obtained from DBT metabolism would accumulate. This intracellular accumulation of toxic sulfite would make *R. erythropolis* activate sulfite oxidoreductase (SOR) to convert it into sulfate. The sulfate would subsequently activate the *cysJ* gene encoding for SR in *R. erythropolis* and the resulting SR would then begin converting both sulfite and sulfate into sulfide for growth. However, for any reason, if any excess sulfite remains, the active SOR would convert it and excrete as sulfate. This is indeed supported by the experimental observations of sulfate as the only excreted sulfur metabolite and in small amounts.[3]

(ii) Enzyme compartmentalization. Alternately, it is also possible that the enzymes involved in the sulfite-to-biomass conversion are compartmentalized in the bacterial cell in a location distinct from that of the Dsz enzymes. In this case, even though it makes energetic sense to feed sulfite from the 4S pathway directly into biomass production, the reality is that the production of sulfite by Dsz enzymes creates metabolic stress that is relieved by converting sulfite to sulfate *via* SOR (as explained previously) so that the sulfur can be routed to the enzyme complex equipped to incorporate it into biomass.

The conversion of all, or some, of the sulfite to sulfate can be understood as a way to avoid intracellular concentrations of sulfite from causing toxicity, but it is not obvious why sulfate formed from sulfite oxidation would be exported outside the cell. Keeping sulfate intracellular so that it can be incorporated into biomass avoids any energy expenditures associated with the exporting and then re-importing sulfate, yet sulfate is found extracellularly in desulfurization-competent cultures.[3,25–27] Moreover, in cases of co-cultures, *R. erythropolis* excretes sulfate that can be readily consumed to meet the sulfur requirements of other bacteria that do not possess *dsz* genes.[34] It is not known if all or only some of the sulfur liberated from the metabolism of DBT by the 4S pathway is released/available as extracellular sulfate, but when co-cultures of desulfurization-competent and desulfurization-deficient bacterial strains are grown with DBT serving as the sole source of sulfur, the population of the desulfurization-deficient culture can exceed the population of the desulfurization-competent culture by ratios as high as 1000-to-1.[34]

This suggests that the entirety of sulfur derived from DBT may be converted from sulfite to sulfate and then sulfate is exported outside the cell before being imported again and incorporated into *R. erythropolis* biomass. The observation by Omori *et al.*[26] that adding barium chloride to the growth medium of desulfurization-competent cultures can stimulate the metabolism of the 4S pathway is a further confirmation that sulfite from the 4S pathway is converted into extracellular sulfate before being incorporated into biomass. The explanation why sulfate is found extracellularly may be related to enzymatic compartmentalization, and it certainly merits further investigation.

Another unresolved topic in the metabolism of sulfur by *R. erythropolis* is what enzyme(s) and pathway(s) are used in converting sulfite to sulfate and extracellular sulfate into biomass. Fig. 2 shows the pathways for the metabolism of sulfur from DBT to various metabolites. Since sulfite is one step closer to being incorporated into biomass than sulfate, it would seem to be advantageous for the 4S pathway to produce sulfite rather than sulfate. Furthermore, the reduction of sulfite directly into sulfide seems less energy intensive than *via* sulfate formation. Therefore, an understanding of sulfite-to-sulfate inter-conversions is also essential.

Sulfite–sulfate inter-conversions in DBT metabolism

We investigated the possible metabolic routes for the inter-conversion of sulfite to sulfate and five metabolic pathways were found as shown in Fig. 3. Of these routes, the enzymes (marked green) for pathways II, and III have been identified[33] to be present in *R. erythropolis* based on its latest genome annotations, and our BLASTp analyses revealed the possible ORFs encoding for enzymes (marked red) of pathways I, IV and V in the genome sequence of *R. erythropolis*. As seen in Fig. 3, the pathways differ in the energy and cofactor requirements. We used our genome scale *in silico* model to investigate the preferred path for conversion of sulfite to sulfate. With all five paths active, our *in silico* cell seems to prefer paths based on ATP need. With zero ATP needed, pathway V (EC 1.8.4.9) is the most preferred, while pathway III (EC 1.8.99.2) and pathway IV (EC 1.8.4.10) are preferred over pathway II (EC 1.8.4.8). That is, III is taken, only when V is blocked. If pathways V and III are blocked, cells utilize sulfate *via* IV, and II is taken only when pathways I, III, IV, and V are all blocked. ATP requirement seems to be the key, as the ATP needed is the highest for pathway II and zero for pathway V.

The five pathways shown in Fig. 3 have different directionalities. It may be that one of the pathways is used for the conversion of intracellular sulfite into extracellular sulfate, while another pathway is used for the conversion of extracellular

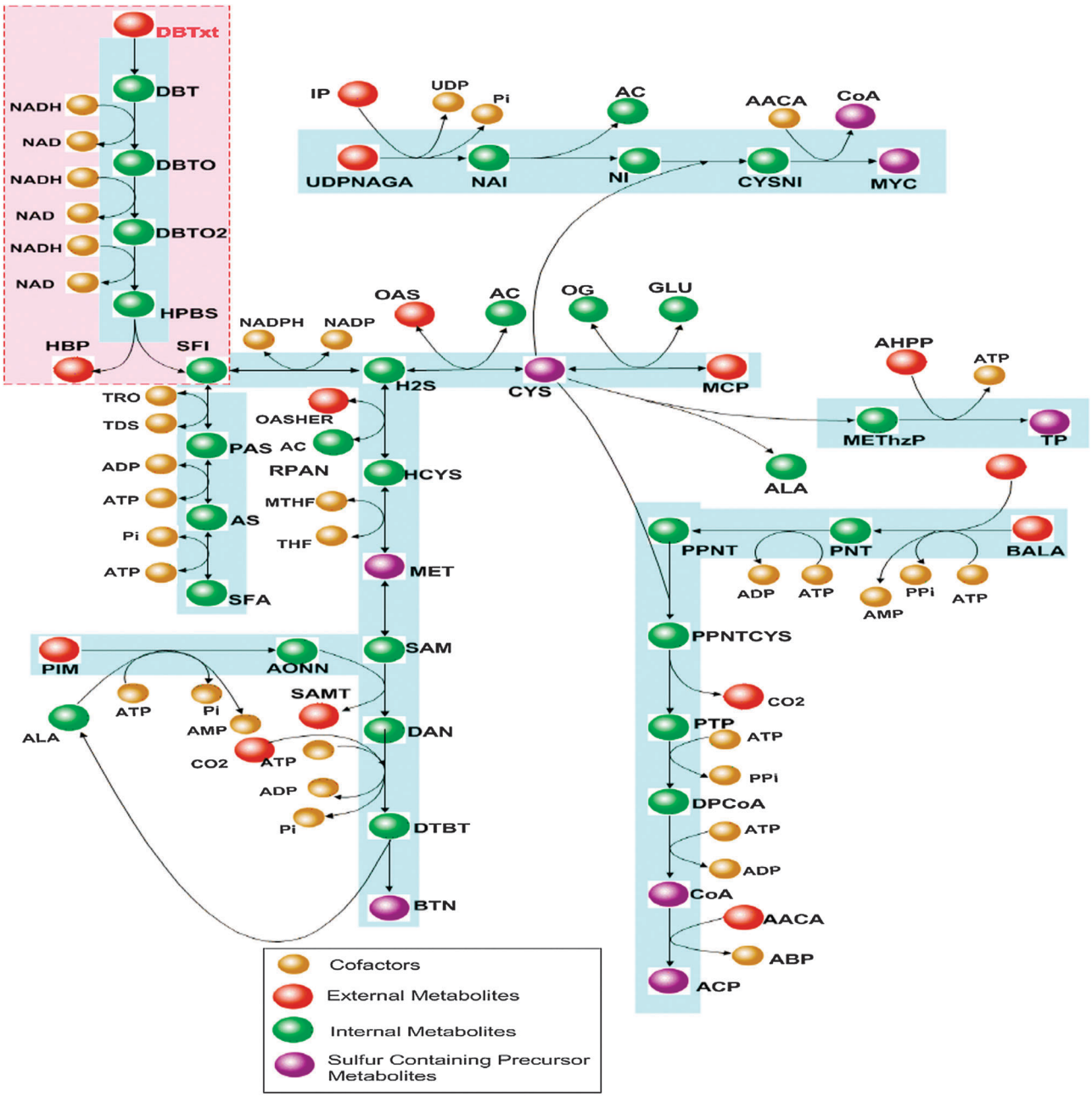


Fig. 2 Schematic for sulfur metabolism adapted from Aggarwal *et al.*[10] For more details please refer to ref. 10. 4S pathway is enclosed within the pink box.

sulfate into intracellular sulfite that leads to incorporation into biomass. Research is needed to explore these possibilities.

Role of SOR

To obtain microbial cultures with higher desulfurization activity, it appears essential that *R. erythropolis* desulfurize DBT in excess of its nutritional requirement for sulfur, and excrete extra sulfite as sulfate. The *in silico* model of Aggarwal *et al.*[33] without SOR confirms this easily. Without an active SOR, it is not possible to force extra flux through the 4S pathway for a fixed carbon source uptake in their steady state model, as sulfite accumulation is not possible due to toxicity. Therefore, to study the role of SOR, we updated and revalidated their genome-scale model by adding the following reaction catalyzed by SOR and simulating the experiments of Omori *et al.*[26] and Folsom *et al.*[18]

$$\text{sulfite} + H_2O + 2\text{ferricytochrome c} \leftrightarrow \text{sulfate} + 2\text{ferrocytochrome c} + 2H+$$

Omori *et al.*[26] observed sulfate excretion along with DBT uptake in ethanol-based media. To simulate these observations, we allowed unlimited ethanol uptake, but fixed the DBT uptake and sulfate production rates at their experimental values to compute maximum cell growth rates. An active

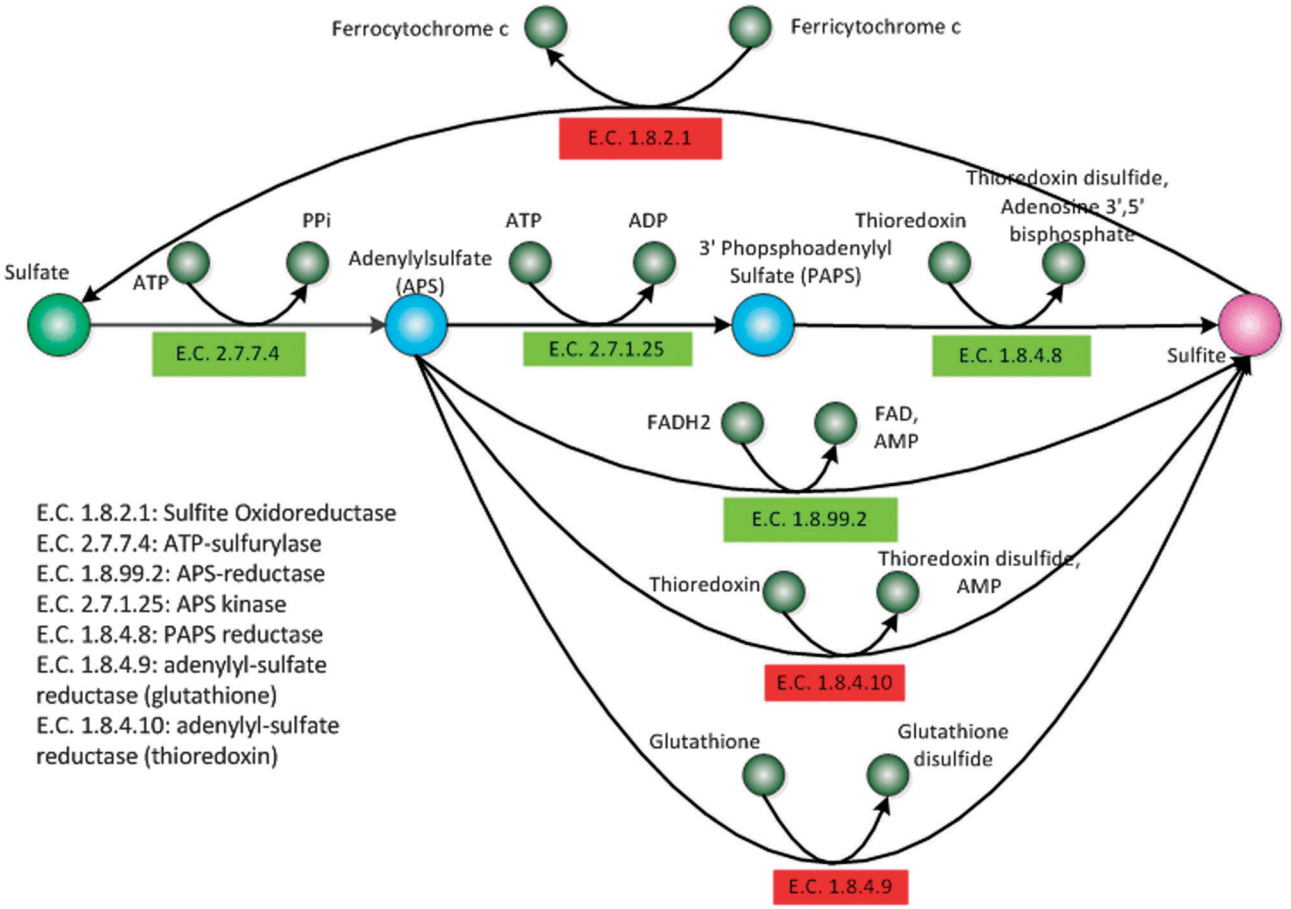


Fig. 3 Schematic showing pathways for oxidation of sulfite to sulfate with associated enzymes.

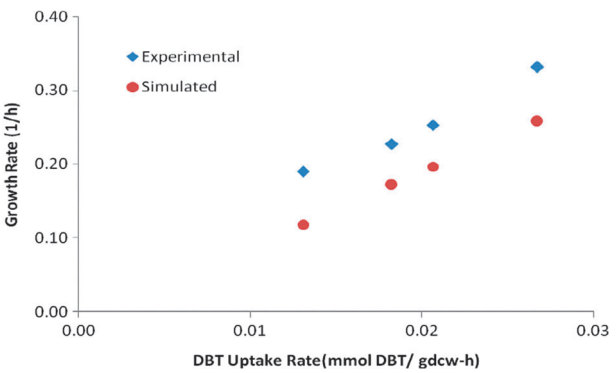


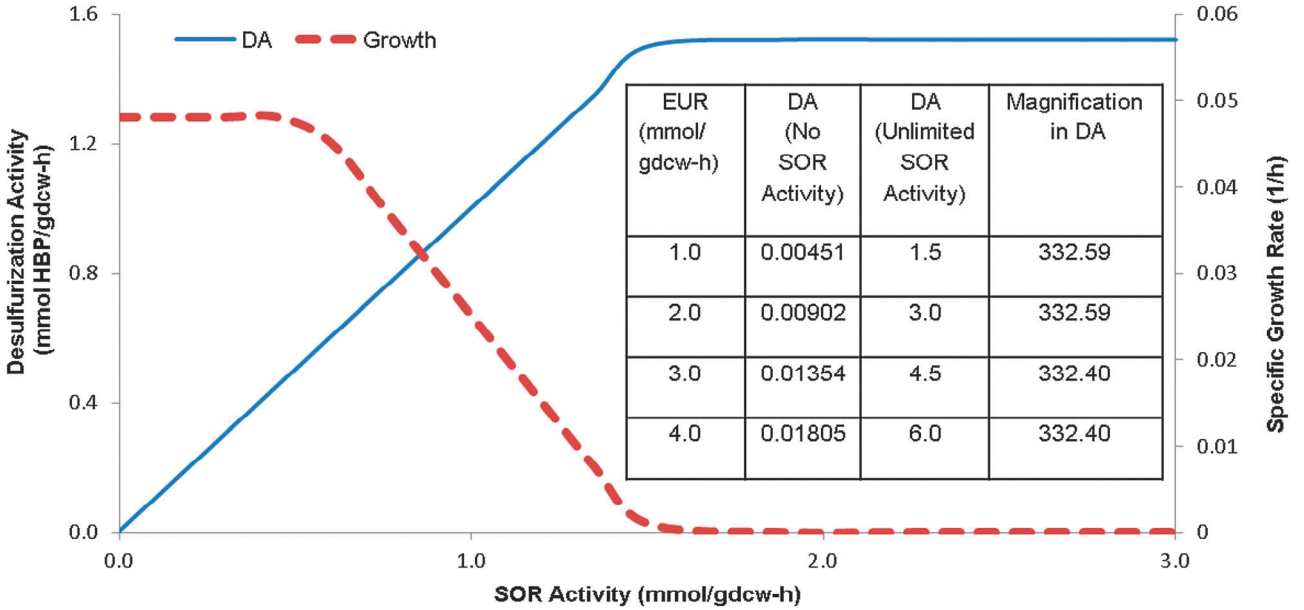
Fig. 4 Cell growth *vs.* DBT uptake for the data from Omori *et al.*[23] The simulations involve fixing DBT uptake rates and sulfate production rates at their experimental values and then maximizing cell growth.

SOR enables sulfate formation and thus subsequent excretion. Fig. 4 shows that our cell growth predictions are in reasonable agreement with experimental measurements of Omori *et al.*[23]

Folsom *et al.*[18] studied DBT desulfurization using *R. erythropolis* I-19, a recombinant strain of *R. erythropolis* IGTS8 containing multiple copies of *dsz* genes. They observed that I-19 exhibited desulfurization rates (5.0 μmol gdcw^{-1} h^{-1}) that were nearly 25 times those exhibited by IGTS8 (0.1–0.2 μmol gdcw^{-1} h^{-1}). In the absence of uptake rates and other data in Folsom *et al.*[18] we proceed to estimate the maximum possible desulfurization activities using our model.

We first study the impact of SOR on desulfurization activity (DA) using our *in silico* model.[33] We fix an arbitrary ethanol uptake rate (say 1 mmol gdcw^{-1} h^{-1}). Then, we set SOR activity (sulfate formation flux) to zero and compute the maximum cell growth rate as 0.048 h^{-1}. At this growth, DA is 4.5 × 10^{-3} mmol HBP gdcw^{-1} h^{-1}, which is the maximum DA for the wild type strain with zero SOR activity. It represents growth related sulfur requirements for *R. erythropolis*. Since our main goal is to remove sulfur from DBT beyond these growth needs, we now allow unlimited *dsz* gene activity, and maximize DA at various SOR activity levels for the ethanol uptake of 1 mmol gdcw^{-1} h^{-1}. As seen in Fig. 5, DA increases linearly with SOR activity, as the active SOR provides a mechanism for removing the surplus sulfite. Fig. 5 clearly suggests that SOR controls DA in the absence of other factors such as *dsz* genes.

Fig. 5 also shows that DA levels off at about 332 times its base value at the zero SOR activity, once SOR activity crosses a threshold. Thus, the maximum possible magnification in DA attainable by increasing the copy numbers of the *dsz* and SOR genes seems to be 332 at an ethanol uptake of 1 mmol gdcw^{-1} h^{-1}. While the maximum DA increases with ethanol uptake (1–4 mmol gdcw^{-1} h^{-1}), its magnification from the base level remains nearly unchanged. Since Omori *et al.*'s[23] experiments point to a non-zero activity of SOR in the wild type *R. erythropolis*,



EUR (mmol/ gdcw-h)	DA (No SOR Activity)	DA (Unlimited SOR Activity)	Magnification in DA
1.0	0.00451	1.5	332.59
2.0	0.00902	3.0	332.59
3.0	0.01354	4.5	332.40
4.0	0.01805	6.0	332.40

Fig. 5 Plot showing effects of SOR activity on desulfurizing activity (DA) and specific growth rate for ethanol uptake rate of 1 mmol gdcw^{-1} h^{-1}. The table shows maximum DA achievable for different uptake rates (1–4 mmol gdcw^{-1} h^{-1}) with zero or unlimited SOR activity.

it seems that the highest possible magnification in DA in the wild type *R. erythropolis* must be lower than the 332 with unlimited copy numbers of *dsz* and SOR genes. In I-19 *R. erythropolis* with an elevated *dsz* copy number, Folsom *et al.*[18] observed only a 25-fold increase in DA. This is much lower than the 332 predicted by our model, and suggests that the SOR activity in Folsom's experiments was very likely limited.

Fig. 5 also shows the impact of SOR activity on cell growth. For DA $\leq$ 0.46 mmol HBP gdcw^{-1} h^{-1} (magnification of about 120 from the base DA), cell growth remains unaffected at its maximum of 0.048 h^{-1}. However, as DA increases beyond this threshold, cell growth decreases linearly with SOR activity, until it becomes zero at the maximum possible DA. This interaction between cell growth and DA can be explained as follows. Initially, ethanol supply is sufficient for the cofactor needs of both cell growth and DA. When DA increases to 120 times its base level, ethanol supply becomes limiting. Cell growth must now compete with DA for its cofactor needs. Thus, it continues to reduce with subsequent increase in DA, until it becomes zero at the maximum DA level. This observation is interesting for obtaining a strain that has lower growth rate, but higher DA. We will discuss a strategy to design such a strain later in this article.

Next we used our *in silico* model to simulate Folsom *et al.*[18] experiments with glucose as a carbon source. We observed that the magnification in DA due to increased SOR activity was up to a factor of 221 with glucose (1–4 mmol gdcw^{-1} h^{-1}) (data not shown) which is much lower than that observed with ethanol (332). This also points to the critical role that the carbon source and its metabolism may play in limiting DA by way of NADH regeneration.[33]

Role of SR

As our main aim is to remove sulfur from DBT by the action of *R. erythropolis* and not cell growth *per se*, it is desirable to have recombinant strains that can show higher levels of DA at reduced growth rates. As discussed earlier, while SOR converts sulfite into sulfate, SR assimilates it into biomass. As such, the flux through SR directly affects the growth (Fig. 6) by limiting the supply of sulfur for biomass synthesis. Our earlier simulations showed that the improvement in DA beyond 120 fold is accompanied by a reduction in growth for a fixed ethanol uptake. Therefore, limiting the SR activity could divert sulfite from biomass to sulfate. To examine the effect of lowering SR activity on DA with unrestricted SOR activity, we fixed ethanol uptake at 1 mmol gdcw^{-1} h^{-1} as earlier, and maximized growth. At the maximum cell growth, SR activity is the highest at 4.51 $\times$ 10^{-3} mmol gdcw^{-1} h^{-1}. Then, keeping ethanol uptake at 1 mmol gdcw^{-1} h^{-1}, we decreased the flux through SR (0–4.51 $\times$ 10^{-3} mmol gdcw^{-1} h^{-1}) and maximized DA. Fig. 6 shows that DA increased, as flux through SR decreased. This suggests that an efficient desulfurizing strain of *R. erythropolis* can be obtained by using higher copy numbers of *dsz* and SOR genes with a reduced activity of SR.

Combined effect of SOR and SR activities

We next examined the combined effects of SR and SOR activities on DA. For this, we performed five sets of simulations with a fixed ethanol supply of 1 mmol gdcw^{-1} h^{-1}. In each set, we fixed the SR activity at a percentage (0, 25, 50, 75, and 100%) of the maximum *i.e.*, 4.51 $\times$ 10^{-3} mmol gdcw^{-1} h^{-1} obtained from Fig. 6. For each SR activity, we varied the maximum SOR activity between 0 and 2 mmol gdcw^{-1} h^{-1}. In each simulation run, we maximized DA. As seen in Fig. 7, the highest DA achievable improves with decreasing SR activity and increasing SOR activity.

SOR activity

Our hypothesized central role of SOR in limiting desulfurization led us to explore the factors that may limit or impact its activity.

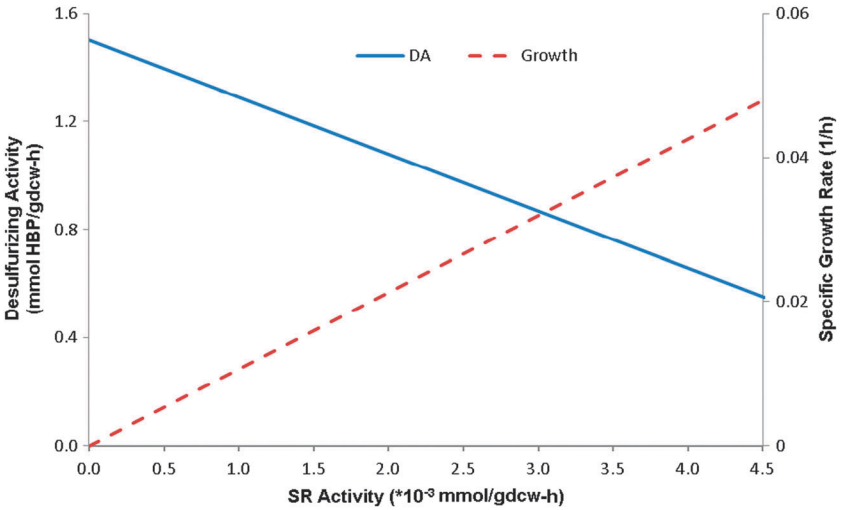


Fig. 6 Effect of SR activity on desulfurizing activity and specific growth rate for an ethanol uptake rate of 1 mmol gdcw^{-1} h^{-1} with unlimited SOR activity.

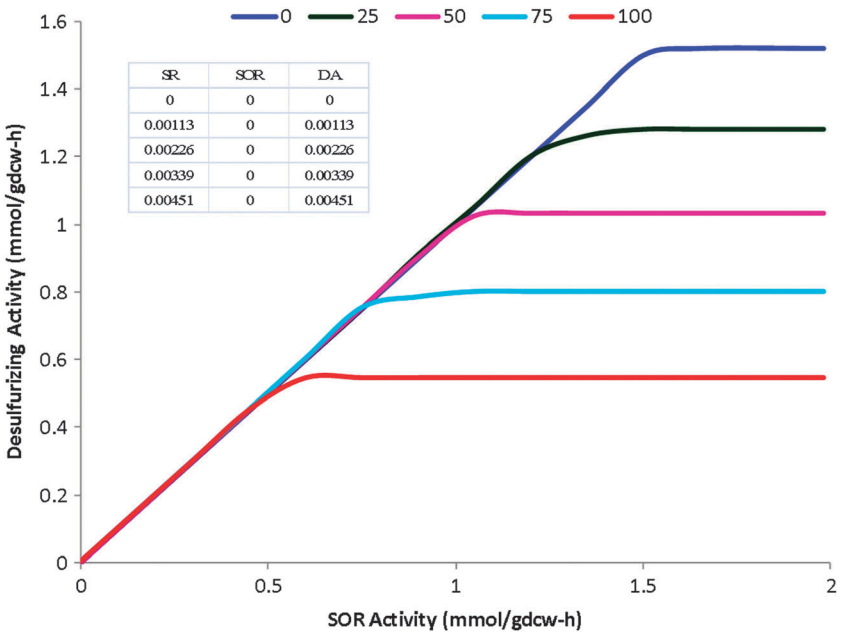


Fig. 7 Effect of simultaneous variations in SR and SOR activities on desulfurizing activity. Each plot is for a fixed SR activity as a per cent of the maximum SR activity. Note that, when SOR activity = 0, the DA = SR activity as shown in the figure. However, this variation in DA with SR activity for SOR activity = 0 is not evident in the plot due to the difference in scale.

Kappler and Dahl[29] reported that sulfate non-competitively inhibits SOR. Omori *et al.*[27] observed enhanced desulfurization and improved growth by removing sulfate from the medium. It is also reported[29,30] that pH has a strong effect on SOR activity. The activity is the highest at pH = 7.5, zero at pH = 3, and decreases as pH decreases.[29] Thus, we decided to focus on the two most critical factors (sulfate and pH) affecting SOR activity.

Effect of sulfate

Some studies[2] have reported sulfate to retard DA. Li *et al.*[35] observed that DA decreased with increasing sulfate presence. They proposed the binding of some repressor protein to the *Dsz* promoter (which lies upstream of *dszA*) as a possible explanation. Later, Franchi *et al.*[23] reported that a recombinant strain with a combination of strong promoters that are not regulated by sulfate showed improved DA. However, when they cultured these strains in the presence of sulfate, DA was still 50% lower than that in the absence of sulfate. They conjectured that some additional sulfate-regulated factors may be controlling desulfurization. In addition, they observed that NADH in the cell extracts of their desulfurizing strains depleted more rapidly in the presence of DBT than sulfate. They suggested that sulfate was lowering the activity of DszD (oxidoreductase) that provides NADH for the 4S pathway, as Hirasawa *et al.*[21] had already shown that sulfate has no effect on the transcription from the *dszD* promoter.

Recently, Aggarwal *et al.*[10,33] proposed an alternate/additional explanation for the effect of sulfate on desulfurization. They argued that any repression effects by sulfate notwithstanding the cell naturally prefers sulfate over DBT. By using sulfate instead of DBT, the cell saves NADH for other growth-related metabolic activities. Indeed, the observations of Franchi *et al.*[23] strongly support this explanation. Aggarwal *et al.*[10] also used the same argument to explain the higher cell growth with sulfate than DBT.[11]

In addition to the above argument of Aggarwal *et al.*,[10] we now propose the inhibitory effect of sulfate on SOR as an additional contributing factor for lower cell growth and reduced DA. The removal of sulfate from the medium by Omori *et al.*[27] prevented the inhibition of SOR activity by sulfate, which resulted in enhanced desulfurization and improved growth. Clearly, the removal of non-toxic sulfate cannot directly increase cell growth, but as discussed earlier, sulfate can non-competitively inhibit SOR activity,[29] which may lead to the accumulation of toxic sulfite and hence lower growth. The cell may also naturally limit DBT uptake to its bare minimum to slow the accumulation of toxic sulfite. That means DA decreases.

Effect of pH

Several studies[3,12,26,36] have reported that decreased medium pH decreases DBT desulfurization by *R. erythropolis*. Furthermore, Wang *et al.*[36] and Olmo *et al.*[9] observed that medium pH strongly affected desulfurization, as well as cell growth. A medium pH of 6.5 seemed the best for growth, while 6.5–6.0 was optimum for desulfurization. When pH was not controlled at its initial value of 6.5, it decreased gradually with substantial reductions in both growth and desulfurization. We can explain the above using the activity of SOR. Since SOR activity decreases with decreasing pH, the conversion of sulfite to sulfate slows and sulfite begins to accumulate in the cell. The toxicity of accumulating sulfite reduces cell growth, which in turn reduces its need for sulfur. Furthermore, the cell's reduced ability to remove sulfite forces it to import only the sulfur needed for growth. All these lead to reduced DA. Clearly there may be other effects of pH on cell metabolism besides the activity of SOR, but this is an example of hypotheses for improving DA that can be generated by metabolic flux analysis, and subsequently tested by experimental investigation.

Materials and methods

Experimental measurements

Our primary source of experimental data is from Omori *et al.*[26] They measured pH, cell growth, DBT concentration, HBP concentration, and sulfate concentration at various times during their study on DBT desulfurization by *R. erythropolis* SY1.[26,27] They used 5 ml of an ethanol-based sulfur-free minimal medium supplemented with 50 μl of 30 mM DBT–ethanol solution at 30 °C to examine the activity of growing cells. For analyzing and predicting various literature observations qualitatively and quantitatively, we used the genome-scale metabolic model[33] of *R. erythropolis* as the basis. We employed the technique of flux balance analysis[37] to analyze the intracellular flux distributions from our model. As discussed later, we also enhanced our model to include additional functionalities.

Flux balance analysis

Flux balance analysis is based on the assumption that metabolic transients are typically rapid compared to cell growth and environmental changes.[38,39] As such, mass balances on various metabolites give us $S \cdot v = b$, where S is the $(m \times n)$ stoichiometric matrix of the reactions, m is the number of metabolites, n is the number of reactions, v is the $(n \times 1)$ vector of reaction fluxes, and b is the $(m \times 1)$ vector of metabolite fluxes. The fluxes are zero (nonzero) for intracellular (extracellular) metabolites. These linear mass balance equations ($S \cdot v = b$) combined with an optimization objective[40] that represents the cellular goal results in a linear optimization model that can be solved using a variety of commercial software. A linear objective function given by $Z = c_1 v_1 + c_2 v_2 + \cdots + c_n v_n$ is used widely in the literature, where v_j is the flux of reaction j and c_j is a constant. To simulate a given experimental scenario by our model, we imposed suitable bounds on or assigned values to some reaction fluxes. In this work, we used MetaFluxNet,[41] which is a metabolic network management software tool.

Developing cultures with improved desulfurization activity

Our above analyses and discussion have highlighted the central and critical roles of sulfate, pH, and SOR in desulfurization. We hypothesize that apart from the copy number of *dsz* genes and supply of NADH, the activity of SOR limits the extent of DBT desulfurization by *R. erythropolis*. Thus, efforts at increasing the desulfurization rate in biocatalysts may benefit by focusing on SOR. While increasing the expression and/or copy number of genes encoding SOR is obviously essential, several other factors must be kept in mind. First, sulfate must be continuously removed from the medium, because elevated concentrations of sulfate will inhibit SOR activity and sulfate will be used in preference to DBT as the source of sulfur to support cell growth. Second, medium pH must be maintained at 6.5 to avoid the inhibition of SOR at lower pH values. Last, adding molybdenum salts may help, as SOR utilizes molybdenum as a cofactor.[29]

Another possibility for improved desulfurization activity suggested by our *in silico* analyses is the simultaneous decrease in SR activity and increase in SOR activity. SR is essential for the utilization of sulfite or sulfate to support cell growth, so deleting the *cysJ* gene would be lethal. But decreasing the expression of the *cysJ* gene to achieve lower SR activity would effectively starve cells for sulfur and result in an increased rate of utilization of DBT provided that elevated levels of SOR are maintained to avoid the toxic accumulation of sulfite. This approach should result in desulfurization of biocatalysts that have a slower growth rate, but higher desulfurization activity.

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