Hierarchical Classification of Enzyme Promiscuity
Using Positive, Unlabeled, and Hard Negative Examples

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Abstract
Despite significant progress in sequencing technology, there are many cellular enzymatic activities that remain unknown. We develop a new method, referred to as SUNDRY (Similarity-weighting for UNlabeled Data in a Residual HierarchY), for training enzyme-specific predictors that take as input a query substrate molecule and return whether the enzyme would act on that substrate or not. When addressing this enzyme promiscuity prediction problem, a major challenge is the lack of abundant labeled data, especially the shortage of labeled data for negative cases (enzyme-substrate pairs where the enzyme does not act to transform the substrate to a product molecule). To overcome this issue, our proposed method can learn to classify a target enzyme by sharing information from related enzymes via known tree hierarchies. Our method can also incorporate three types of data: those molecules known to be catalyzed by an enzyme (positive cases), those with unknown relationships (unlabeled cases), and molecules labeled as inhibitors for the enzyme. We refer to inhibitors as hard negative cases because they may be difficult to classify well: they bind to the enzyme, like positive cases, but are not transformed by the enzyme. Our method uses confidence scores derived from structural similarity to treat unlabeled examples as weighted negatives. We compare our proposed hierarchy-aware predictor against a baseline that cannot share information across related enzymes. Using data from the BRENDA database, we show that each of our contributions – hierarchical sharing, per-example confidence weighting of unlabeled data based on molecular similarity, and including inhibitors as hard-negative examples – contributes towards a better characterization of enzyme promiscuity.

1. Introduction
Characterizing activities of enzymes promises to play a critical role in advancing biological and biomedical engineering applications. Enzymes are traditionally assumed specific, acting on a specific molecule. The Enzyme Commission (EC) number nomenclature, which assigns EC numbers to enzymes based on experimental evidence of enzyme-catalyzed reactions [1], describes such specificity. Four numbers separated by periods (e.g. 1.2.1.75) provide a tree-structured hierarchical classification of enzyme function. At the top level, there are 7 potential classes of enzymes (numbered 1-7). Classes are further divided into sub-classes, sub-subclasses, and finally individual enzymes at the leaf level of the hierarchy. Despite widespread use, EC classification does not reflect the diverse range of molecules that enzymes catalyze. For example, L-lactate dehydrogenase (LDH), associated with EC number 1.1.1.27, is responsible for lactic acid formation in human muscle cells during stressful exercise as well as in bacteria during fermentation of milk to yogurt. However, LDH also transforms substrates other than lactic acid, including 2-hydroxybutyrate, 2-hydroxy-3-methylbutanoate, and other structurally similar molecules. Importantly, many enzymes, if not all, have promiscuous activities such that they act on substrates other than those they evolved to transform [2-5]. Despite efforts in cataloguing enzyme activities on various substrates in databases, comprehensive characterization of enzyme promiscuity remains elusive.

Developing tools to identify enzymes that act on a specific molecule can augment existing catalogued enzymatic activities. Techniques such as Metro [6, 7], Metaprint2D-react [8], and PROXIMAL [9], are specific to Cytochromes P450 enzymes, which are liver enzymes with broad specificity. Substrate similarity methods investigate the similarity between a query molecule and the native substrate that is known to be catalyzed by the enzyme [10]. Similarity can be computed using subgraph isomorphism [11] or molecular fingerprints, which represent complex molecular structure via binary feature vectors of predetermined size (e.g., PubChem
fingerprints [12], Extended-Connectivity (ECFP) [13]). There is no consensus however on a similarity level that deems a query molecule sufficiently similar to a native substrate. Our work improves on these prior efforts by training and evaluating classifiers that identify enzymes that act on a specific molecule. We create such classifiers for over 1000 distinct EC numbers. This is a huge increase over previous efforts [14] that targeted 4 specific enzymes and where active learning was applied to recommend substrates for experimental testing to improve classification accuracy. Instead of requiring expensive experimental testing, our approach judiciously leverages enzyme-substrate pairs catalogued in existing databases and makes use of unknown enzyme-substrate interactions.

We present a new technique, SUNDRY (Similarity-weighting for UNlabeled Data in a Residual HierarchY), for training predictors specific to each enzyme that can share information using known hierarchical relationships of enzymes. Our technique casts the problem of predicting the activity an enzyme on a query substrate as a multi-label hierarchical classification problem (for a survey of hierarchical classification problems and methods, see [15]). Probabilistic predictions are made at each node of EC tree hierarchy. Each node takes as input a given query molecule fingerprint and the prediction of its parent node for that molecule. As output, the node produces a probability that enzymes belonging to it will act on the query molecule. Predictors are trained sequentially from top level to leaf-level. Each node is trained to predict the residual correction needed from its parent to produce a more accurate prediction.

A major challenge in training any classifier for substrate promiscuity concerns the availability of labeled examples. The BRENDA database [16] is the largest database cataloguing enzymatic reactions and their substrates. Lists of substrate molecules for each enzyme provides positive examples. The BRENDA database also catalogues inhibitor molecules. Inhibitors bind to enzymes and prevent them from catalyzing reactions. As they bind with enzymes, inhibitors might be considered ‘closer’ to the positive molecules and thus be more difficult (harder) to classify. Including such ‘hard negative’ examples can fine-tune the decision boundary between positives and negatives [17]. However, experimentally confirmed negative examples that are not inhibitors are not systematically catalogued and are mostly unavailable.

We overcome this lack of available negative data by developing a confidence-weighted labeling procedure for unlabeled examples. We first identify molecules that are specifically unlabeled for each enzyme (not in the positive list nor in the inhibitor list). We treat each such example as negative, because negative interactions generally far outnumber positive interactions in nature. However, we apply a probabilistic weight to each such example to reflect our confidence in the negative label, deriving the weight from the molecule’s structural similarity score to the nearest positive example.

Our proposed method has three key contributions over previous methods for substrate promiscuity prediction that help us overcome label scarcity problems: hierarchical sharing across enzymes that leverages the EC tree structure, training with inhibitors as hard negatives, and per-example confidence weighting of unlabeled data based on molecular similarity. We evaluate our approach on prediction tasks for over 1000 enzymes. Comparisons against non-hierarchical baselines or baselines without careful negative example curation show that each contribution improves prediction quality.

2. Methods
2.1 Data Collection
All positive and inhibitor molecules were collected from BRENDA, excluding co-factors because these metabolites are common across enzymatic reactions. The MACCS fingerprint was used to represent each molecule, with 167 binary features [18]. Not all compound names in BRENDA could be mapped to a specific molecular structure. By the end of the conversion process from names in BRENDA to MACCS fingerprints, we identified 34,122 positive pairings between molecules and EC numbers, based on 9,839 unique molecules. We also identified 69,374 inhibiting interactions, based on 14,347 unique inhibitors. Some individual enzymes had limited positive data. We therefore predicted promiscuity for leaves that had a minimum of 10 positive examples. There was sufficient data to train classifiers for 1007 distinct EC numbers. Only 927 of these enzymes had known inhibitors. As there were not many enzymes classified for the recently established top level class (number 7), we only used data for classes 1-6.
The data was further organized in a tree hierarchy to match the structure of the EC nomenclature. There were 6 nodes at the class level (top of hierarchy), 50 nodes at sub-class level, 146 nodes at sub-subclass level, and 1007 leaf nodes (distinct EC numbers). At all non-leaf nodes in the hierarchy, positive examples consisted of the union of all positive examples at any child. Similarly, inhibitor (hard negative) data consisted of inhibitors at any child unless already labelled positive due to a positive label from any other child node. At each node, any molecule that is not positive nor an inhibitor is considered unlabeled. All available data were sampled to obtain balanced label representation (otherwise unlabeled examples would dominate and could prevent effective learning). At each node, we kept all positive examples as well as an equal number of negative examples. At most one third of the negatives were sampled uniformly at random from the inhibitors (depending on availability). The remaining negatives were sampled from the unlabeled examples. This balanced dataset was then used for both training and evaluation, as described in subsequent sections. A pre-specified number of inhibitors (one third) were set aside for independent evaluation.

2.2 Models
2.2.1 Baseline Model
A Random Forest (RF) binary classifier [19] was built for every leaf in the tree (distinct EC number). Each RF classifier was composed of 50 decision trees, each trained to optimize the Gini metric and allowed to select from a random subset of features, of size proportional to the square root of the number of features, at each internal decision node. We trained and evaluated a separate RF classifier on each node’s data using 5-fold cross-validation, recording the mean and standard deviation of performance metrics across the folds. During training, we selected model complexity hyperparameters (the minimum size of any tree’s terminal node) by performing an inner-loop of 5-fold cross-validation grid search. The grid search considered minimum terminal node sizes of 1, 5, 10, 20, 50, 100, and 200, selecting the value with best average precision score (averaged across the five inner folds). Overall our nested cross-validation approach allowed better use of limited data compared to simply leaving some data aside for testing.

2.2.2 Hierarchical Model
Our proposed hierarchical model uses a tree-like architecture that mimics the hierarchy of the EC nomenclature. We trained a hierarchical cascade of random forests (RFs), with one RF predictor at each node of the tree. Each of the 6 top-level enzyme categories had a root predictor trained to produce probabilistic predictions given binary labeled data. Then, at each lower-level node an RF regressor was trained to predict the residual error from its parent [20]. The overall probabilistic prediction at a node is thus formed by adding its predictions to all preceding levels (thresholding to keep a valid probability in the unit interval). The hyperparameters of the RF classifiers and RF regressors were selected in the same exact way as in the baseline model. We use the same nested cross-validation procedure for testing as for the baseline model. We made sure that the splits were consistent across the tree, to ensure that testing data at any given fold had not been previously used to train any internal node at the same fold that contributes to the prediction.

2.3 Training Methods – Confidence Weighting of Unlabeled data
Providing a per-example confidence weight (a scalar positive value) is a common technique to overcome label balance issues or account for unlabeled data that may unknowingly contain positive examples [21]. We consider every unlabeled example as having a negative label together with a scalar confidence equal to one minus the maximum structural similarity found between the unlabeled molecule and all molecules in the positive set [10]. Similarity is scored using the Tanimoto score for two molecular fingerprints. Each confidence weight associated with the unlabeled set is a scalar between 0 and 1, while the weights for the positives and inhibitors are set to 1. We provide these weights when training RF predictors using the 'sample_weight' keyword argument in Scikit-Learn [22]. During evaluation, we computed weighted averages of performance metrics across examples. We evaluate both a baseline “unit-weighted” preprocessing (all unlabeled examples are negative with confidence 1) and our proposed “similarity-weighted” preprocessing.
3. Results

3.1 Comparing non-hierarchical and hierarchical models using different weighting techniques

We evaluated how the different approaches to weighting unlabeled examples as negatives — unit-weighting or similarity-weighting — affect the performance of the two models — Hierarchical and Baseline (non-hierarchical). We use three metrics to assess performance. First, average precision (AP) isolates how well the classifier performs on examples it has called positive (higher is better). Second, the area-under-the-ROC curve (AUROC) measures the overall balance of specificity and sensitivity (higher is better). Both AP and AUROC, are reported as means and standard deviations from nested cross validation. Finally, we assess the true negative rate (TNR) on a separate heldout set of known inhibitors (higher is better). This assesses more directly the ability to distinguish difficult negative examples. The results are shown in Table 1.

The results show several trends. The two hierarchical models consistently outperform the corresponding non-hierarchical models with respect to all metrics. The similarity-weighted models are consistently better or equal to the models that weight all unlabeled examples equally, both for hierarchical and non-hierarchical models, with the exception of AUROC for the two hierarchical models. In addition, the fact that the TNR results are so low relative to the other metrics indicates that heldout inhibitors are hard to classify, thus supporting the theory that they are hard negative examples.

| Model                  | AP        | AUROC    | TNR on heldout inhibitors |
|------------------------|-----------|----------|---------------------------|
| Baseline + unit-weighted| 0.795 ± 0.100 | 0.829 ± 0.099 | 0.605 ± 0.307 |
| Baseline + similarity-weighted | 0.853 ± 0.081 | 0.840 ± 0.097 | 0.600 ± 0.304 |
| Hierarchical + unit-weighted | 0.925 ± 0.065 | 0.919 ± 0.071 | 0.708 ± 0.283 |
| Hierarchical + similarity-weighted | 0.942 ± 0.056 | 0.916 ± 0.078 | 0.710 ± 0.281 |

Table 1: Results for models trained with inhibitors (hard negatives).

3.2 Impact of training without true negatives (inhibitors)

Next, we evaluate the impact of using the inhibitors as part of our data set. To do so, we trained the models using only positive and unlabeled data (which we include as negative examples with appropriate weights). We then evaluate on the same inhibitor set as in the previous section. Results are shown in Table 2. When comparing the results with and without inhibitors (Table 1 vs Table 2), the TNR when using inhibitors is consistently better (higher) than when training without inhibitors. This shows that using inhibitors during training effectively helps the classifiers fine-tune the decision boundary between positives and negatives.

| Model                  | TNR on heldout inhibitors |
|------------------------|---------------------------|
| Baseline + unit-weighted| 0.495 ± 0.289              |
| Baseline + similarity-weighted | 0.495 ± 0.291             |
| Hierarchical + unit-weighted | 0.555 ± 0.270             |
| Hierarchical + similarity-weighted | 0.567 ± 0.247             |

Table 2: Results of the models trained without inhibitors (hard negatives).

4. Conclusion and Discussion

This paper proposes a novel tool for predicting whether a given enzyme will act on a query molecule. The tool exploits the inherent hierarchical structure of the Enzyme Commission nomenclature to share statistical strength across related enzymes, resulting in large and consistent gains in prediction quality. The tool also exploits vast amounts of unlabeled data but judiciously weights them based on a similarity score to achieve further gains. Finally, incorporating inhibitors during training allowed for better characterization of the negative set. Our results suggest that predicting enzyme promiscuity through machine learning techniques that leverage existing knowledge in databases hold promise to advance biological engineering practices. The work presented here can be improved by exploring alternate hierarchical classification techniques [23] and considering learned representations that better capture molecular structure than binary fingerprint vectors, e.g. [24].
While there are works that use sequences to predict protein function in terms of GO terms [25, 26] and to predict EC numbers [27], the problem solved within predicts enzyme classes that act on a query molecule. This problem is pressing in synthetic biology and biological engineering applications when constructing biochemical conversion routes to synthesize valuable specialty chemicals such as biofuels, solvents, polymers and others [28]. This problem is also important when constructing biodegradation pathways of environmental pollutants and xenobiotics. SUNDRY can be combined with tools for route exploration and construction [29] to allow for novel transformation steps that are not currently documented in existing databases.

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Appendix

A Conversion pipeline from BRENDA names to MACCS fingerprints
Considerable effort was put forth in collecting our dataset for each enzyme in the BRENDA database (Figure 1). Substrates and products of enzymatic reactions are specified in BRENDA using their common names. We first searched common compounds names in the PubChem database, and collected PubChem IDs associated with each compound. We then collected the SMILES (Simplified Molecular Input Line Entry System) of every compound by querying the PubChem IDs in PubChem. Lastly, we used the RDKit to convert SMILES patterns into MACCS fingerprints. Each MACCS fingerprint consists of 167-bit vector, where each vector entry indicates the presence or absence of a particular molecular feature. Some data was lost at each step of the conversion.

Figure 1: Pipeline of the conversion from compound names in BRENDA to MACCS fingerprints. At each step, some molecules are lost.